The Molecular Pathogenesis of Ovine Herpesvirus 2

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Declaration

I declare that this thesis has been composed by myself and has not been submitted for any other degree. The work described herein is my own except where otherwise indicated and all work of other authors is duly acknowledged.

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Abstract

Malignant catarrhal fever (MCF) is a lymphoproliferative and inflammatory disease of cattle, deer and other ruminants. It is caused mainly by one of two herpesviruses, alcelaphine herpesvirus 1 (AlHV-1) and ovine herpesvirus 2 (OvHV-2). AlHV-1 can be grown in tissue culture; its complete genome has been sequenced and the virus characterised as a gammaherpesvirus. OvHV-2 cannot be grown in tissue culture and only a small amount of OvHV-2 genome sequence is known. Lymphoblastoid cell lines can be propagated from the tissues of cattle, deer and experimentally infected rabbits with MCF. Some of these cell lines transmit disease. The initial aim of this project was to further characterise these cell lines and to use them as a source for cloning the OvHV-2 viral genome.

The OvHV-2 infected cell lines were found to contain on average 50 copies of viral genome per cell. Gardella gel analysis of OvHV-2 infected rabbit lines showed evidence of substantial levels of viral replication, whilst analysis of an OvHV-2 infected bovine cell line showed the virus genome to be predominantly in the latent form. Analysis of RNA extracted from OvHV-2 infected rabbit cell lines demonstrated evidence of early (ORF 57) and late (ORF 75) gene expression. This prompted examination of cell lysates from OvHV-2 infected rabbit cells, resulting in the first visualisation of an OvHV-2 capsid.

A cosmid library was constructed using genomic DNA extracted from the OvHV-2 infected cattle cell line BJ/1035. Screening the cosmid library led to the generation of overlapping cosmids spanning most of the viral genome. The virus sequence was further extended using splinkerette PCR. Analysis of the viral sequence revealed that the OvHV-2 genome is similar to that of AlHV-1 and the unique "A"genes of AlHV-1 are conserved in OvHV-2. OvHV-2 also contains ORFs not present in the AlHV-1 genome, including an IL-10 homologue. The sequence derived is expected to be an invaluable resource for further study of OvHV-2 pathogenesis.

The OvHV-2 ORFs O7 and O8 were contained in the cosmid sequence. It was hypothesised that the two sequences were spliced together to form a membrane glycoprotein of similar function to gp350/220 of EBV. Evidence of splicing of the O7/O8 sequence was found in O7/O8 transfected tissue cultured cells, however this

splicing appeared incomplete and it was hypothesised that viral factors might be required for correct splicing. Immunofluorescence analysis of OvHV-2 infected rabbit cells using anti-O8 immune sera showed evidence that a high proportion of OvHV-2 infected cells expressed O8. These studies provided a starting point for further investigation of this potentially important region of the OvHV-2 genome.

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Abbreviations

A	Adenosine
aa	Amino acid
AIDS	Acquired immunodeficiency syndrome
AlHV-1	Alcelaphine herpesvirus 1
AP	Alkaline phosphatase
ATP	Adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indoyl phosphate
Bcl-2	B cell lymphoma/leukaemia 2
BoHV-4	Bovine herpesvirus 4
BL	Burkitt's lymphoma
BLHV-1	Bovine lymphotropic herpesvirus 1
BLV	Bovine leukaemia virus
bp	Base pair
BSA	Bovine serum albumin
BVDV	Bovine viral diarrhoea virus
C	Cytidine
CD	Cluster of differentiation
cDNA	Complementary DNA
cfu	Colony forming unit
CKR	Chemokine receptor
CMV	Cytomegalovirus
CprHV-2	Caprine herpesvirus 2
CTL	Cytotoxic T lymphocyte
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTP	Deoxynucleoside triphosphate
dTTP	Deoxythymidine triphosphate

dH_2O	Distilled water
diH_2O	De-ionised water (18 megaohm)
ds	Double stranded
EBER	Epstein-Barr virus-encoded small RNA
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
E. coli	*Escherichia coli*
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetraacetic acid
EGFP	Enhanced green fluorescent protein
EHV-2	Equine herpesvirus 2
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
FCS	Foetal calf serum
FADD	Fas associated death domain
FGARAT	Formylglycinamide ribotide amino transferase
FITC	Fluoroscein isothiocyanate
FLICE	FADD homologous ICE/CED-3-like protease
FLIP	FLICE inhibitory protein
G	Guanosine
g or gp	Glycoprotein
GFP	Green fluorescent protein
GI	Gastrointestinal
GPCR	G protein coupled receptor
GS	Glutathione sepharose
GST	Glutathione S-transferase
HA	Haemagglutinin
HCMV	Human cytomegalovirus
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
HSUR	Herpesvirus saimiri U-RNA
HSV	Herpes simplex virus

HRP	Horseradish peroxidase
HTLV-1	Human T lymphotropic virus 1
HVA	Herpesvirus ateles
HVS	Herpesvirus saimiri
ICAM	Intracellular adhesion molecule
ICP	Infected cell protein
IE	Immediate early
IFN	Interferon
Ig	Immunoglobulin
IF	Immunofluorescence
IIF	Indirect immunofluorescence
IL	Interleukin
IM	Infectious mononucleosis
IPTG	Isopropyl-ß-D-thiogalactopyranoside
ITAM	Immunoreceptor tyrosine base activation motif
kb	Kilobase
KDa	Kilodalton
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma associated herpesvirus
LANA	Latency associated nuclear antigen
LAT	Latency associated transcript
LB	Luria Bertani
LCL	Lymphoblastoid cell line
LCV	Lymphocryptovirus
LGL	Large granular lymphocyte
LMP	Latent membrane protein
MCD	Multicentric Castleman's disease
MCF	Malignant catarrhal fever
MCS	Multiple cloning site
MHC	Major histocompatibility complex
MHV-68	Murine gammaherpesvirus-68
MIP	Macrophage inhibitory protein

MOPS	3-N-morpholino-propanesulphonic acid
mRNA	Messenger RNA
NK	Natural killer
NBT	Nitro blue tetrazolium
NPC	Nasopharyngeal carcinoma
NCAM	Neural cell adhesion molecule like protein
OD	Optical density
Oligo dT	Oligodeoxythymidine
ORF	Open reading frame
Ori	Origin of replication
OvHV-2	Ovine herpesvirus 2
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
Pbl	Peripheral blood leukocyte
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PEL	Primary effusion lymphoma
PFU	Plaque forming unit
PLHV	Porcine lymphotropic herpesvirus
PTLD	Post transplant lymphoproliferative disease
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
RPMI	Rosewell Park Memorial Institute
RRV	Rhesus rhadinovirus
RT	Room temperature
SA-MCF	Sheep-associated MCF
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate
STP	Saimiri transforming protein
SV40	Simian vacuolating virus 40
T	Thymidine
TCR	T cell receptor

TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TBS	Tris buffered saline
TE	Tris EDTA buffer
TEMED	N,N,N',N'-tetraethylmethylenediamine
TIP	Tyrosine kinase interacting protein
TK	Tyrosine kinase
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TPA	12-O-tetradecanoyl phorbol-13-acetate
TR	Terminal repeat
U	Unit
UV	Ultraviolet light
v	Prefix for viral
VN	Virus neutralising
VZV	Varicella zoster virus
WA-MCF	Wildebeest-associated MCF
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indoyl-ß-D-galactoside

Chapter One: Introduction

1.1 The Herpesviruses

Herpesviruses are widely disseminated in nature, having been discovered throughout the spectrum of vertebrates and in at least one invertebrate. In nature, each herpesvirus is closely associated with a single host species. The most extensively studied hosts are infected by several distinct herpesviruses. Approximately 130 herpesviruses have been identified to date (Minson, 2000), however this is thought to be only a small proportion of the number in existence. Herpesviruses are well adapted to their hosts and generally, severe infection is usually observed only in the very young, the foetus, the immunosuppressed, or following infection of an alternative host.

Once a host is infected with a herpesvirus, the virus persists in the host for life as a latent infection. The ability to establish a latent infection is a property shared by all herpesviruses. In the latent state the virus genome persists in the infected cell, however no infectious virus particles are produced and a restricted pattern of virus gene expression is displayed. Latent virus retains the ability to undergo reactivation, at which point viral replication is resumed and infectious virions are produced. It is thought that the establishment of latency allows the virus to avoid the host immune system and therefore is an aid to persistence in the host.

The features of a herpesvirus infection can be illustrated using the alphaherpesvirus bovine herpesvirus 1. This virus is the cause of respiratory disease (infectious bovine rhinotracheitis) and genital disease (infectious vulvovaginitis and balanoposthitis) in cattle. Following primary infection in epithilia of the respiratory or genital tracts, lifelong latency is established in the either the sciatic or trigeminal ganglia respectively. Reactivation of the virus, usually due to stress, leads to asymptomatic shedding of virus. Shedding of virus due to reactivation in latently infected cattle can cause serious outbreaks of disease in naïve cattle; the disease syndrome is often referred to as "shipping fever".

Similarly feline herpesvirus 1 is a common cause of respiratory disease in cats. Although mortality is low in adult cats, the virus can cause fatal pneumonia in kittens. Recovered animals can shed virus in the nasal secretions and lifelong latency is established in the trigeminal ganglion. Inactivated and attenuated live vaccines are commonly used to prevent both these diseases, however although the vaccines

provide protection against clinical disease, they do not prevent infection or the establishment of latency.

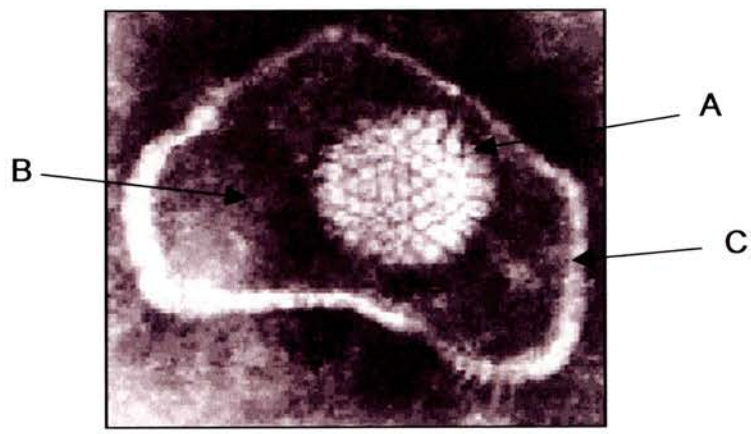
1.1.1 Biological Properties of Herpesviruses

All known herpesviruses share four biological properties (reviewed in Roizman, 2001c).

1. They specify a large array of enzymes involved in nucleic acid metabolism (e.g. thymidine kinase, thymidylate synthase, dUTPase, ribonuclease reductase), DNA synthesis (e.g. DNA polymerase, helicase, primase) and protein processing (e.g. protein kinases).
2. The synthesis of viral DNAs and capsids occurs in the nucleus and capsids are enveloped as they pass through the nuclear membrane.
3. Production of infectious progeny is accompanied by destruction of the infected cell.
4. All herpesviruses studied to date are able to remain latent in their natural hosts. In cells harbouring latent virus the viral genomes take the form of closed circular molecules and only a small subset of viral genes are expressed. Latent genomes retain the capacity to replicate and cause disease upon reactivation.

The primary means of identifying members of the family Herpesviridae has been that of virion structure. A typical herpesvirion consists of a core containing the linear double stranded DNA genome and an icosahedral capsid approximately 100 to 110nm in diameter containing 162 capsomeres. This is surrounded by a structure known as the tegument and an envelope containing viral glycoprotein spikes on its surface (Figure 1.1). The herpesviruses have been further classified into three subfamilies initially on the basis of their biological properties, before the DNA sequence of the virus genome were known. Herpesviruses have been classified into genera, on the basis of DNA sequence homology and antigenic relatedness of viral proteins. Examples of the herpesviruses and their hosts are shown on table 1.1.

Figure 1.1 An Electron Microscopy Image of Herpes Simplex Virus 2



The negatively stained image shows an icosahedral capsid (A) made up of many capsomeres. This is surrounded by a tegument (B) and a glycoprotein envelope (C).

Table 1.1 – Examples of herpesviruses

Name	Host	Disease
Alphaherpesviruses		
Herpes simplex-1	Human	Cold sores, keratitis
Herpes simplex-2	Human	Genital herpes
Varicella zoster virus	Human	Chicken pox and shingles
Bovine herpesvirus-1	Bovine	Infectious bovine rhinotracheitis, Genital disease, abortions
Feline herpesvirus-1	Cats	Feline viral rhinotracheitis
Porcine herpesvirus-1	Pigs	Pseudorabies (Aujzesky's disease)
Equine herpesvirus-1	Horses	Abortion, neurological disease
Gallid herpesvirus-2	Chickens	Marek's disease
Betaherpesviruses		
Human cytomegalovirus	Human	Mononucleosis, multi-system disease in immunocompromised patients
Porcine cytomegalovirus	Pigs	Rhinitis in piglets
Murine cytomegalovirus	Mice	
Human herpesvirus-6	Human	Fever and rash in children
Human herpesvirus-7	Human	Mononucleosis in adults
Gammaherpesviruses		
Epstein-Barr virus	Human	Infectious mononucleosis, Burkitt's lymphoma, PTLD, nasopharyngeal carcinoma,
Kaposi's sarcoma herpesvirus	Human	Kaposi's sarcoma, Body cavity lymphomas, MCD
Herpesvirus pan	Chimpanzee	
Herpesvirus papio	Baboon	
Equine herpesvirus-2	Horses	? Respiratory disease in foals
Bovine herpesvirus-4	Cattle	? Conjunctivitis, metritis, mastitis
Herpesvirus saimiri	Squirrel monkey	Fatal lymphoproliferations in cottontail rabbits & new world monkeys e.g. marmosets
Herpesvirus ateles	Spider monkey	Lymphomas in new world primates
Rhesus rhadinovirus	Rhesus monkey	Lymphoproliferative disease in immunosuppressed host
Murine gammaherpesvirus-68	Murid rodents	Lymphomas
Porcine lymphotropic herpesvirus-1	Pigs	
Alcelaphine herpesvirus-1	Wildebeest	Malignant catarrhal fever in cattle, deer and other ruminants
Ovine herpesvirus-2	Sheep	

1.1.2 The Alphaherpesviruses

Members of this subfamily have a variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells and the capacity to establish latent infections primarily in sensory ganglia. This subfamily contains the genera *Simplexvirus* (e.g. Herpes simplex virus-1), *Varicellovirus* (e.g. Varicella zoster virus), Marek's disease virus and Infectious laryngotracheitis virus.

1.1.3 The Betaherpesviruses

Members of this subfamily have a restricted host range, a long reproductive cycle and grow slowly in culture. Infected cells frequently become enlarged, forming cytomegalia. Latent infections can be established in secretory glands, lymphoreticular cells, kidneys, and other tissues. This subfamily contains the genera *Cytomegalovirus* (e.g. Human cytomegalovirus), *Muromegalovirus* (e.g. Murine cytomegalovirus), and *Roseolovirus* (e.g. Human Herpesvirus-6).

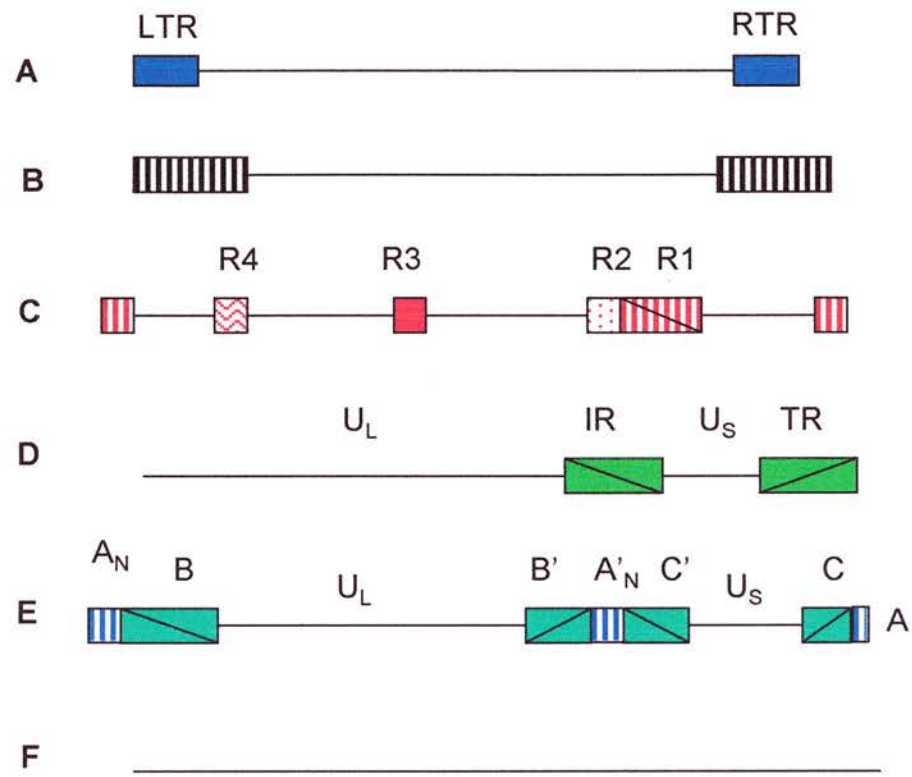
1.1.4 The Gammaherpesviruses

Members of this subfamily have a narrow host range. The experimental host range is generally limited to the family or order to which the natural host belongs. All members of the family replicate *in vivo* in lymphoblastoid cells and some also cause lytic infections in epithelial or fibroblast cells. The gammaherpesviruses establish latency in lymphoid tissue. This sub-family contains the genera *Lymphocryptovirus* (LCV), the prototype of which is Epstein-Barr Virus (EBV), and *Rhadinovirus*, the prototypes of which are Ateline herpesvirus-2 (AtHV-2) and Herpesvirus saimiri (HVS).

1.1.5 Herpesvirus Genomes

Herpesvirus DNA genomes are linear and double stranded. The length varies from 120bp to 250 kb and the base composition varies from 31% to 75% GC content. The viral DNA circularises on release from the capsid into the nucleus of infected cells. Herpesvirus genomes usually contain a number of reiterated sequences. The arrangement of the repeated sequences either at the termini or internally, results in a number of different genome structures (Figure 1.2).

Figure 1.2 Sequence Arrangements of Herpesvirus Genomes



The sequence arrangements of the six classes of viral genomes of the herpesvirus family are shown. In group A genomes, e.g. Channel catfish herpesvirus, sequence from one terminus is directly repeated at the other terminus. In group B genomes, such as herpesvirus saimiri, Alcelaphine herpesvirus-1, Kaposi's sarcoma herpesvirus, the terminal sequence is directly repeated many times at both termini. The number of reiterations at the termini may vary. In group C genomes, such as in Epstein-Barr virus (EBV), the genome contains internal unrelated repeat sequences greater than 100bp, named internal repeats (IR), as well as terminal repeats. In group D viral genomes, such as Varicella zoster virus, the sequence at one terminus (TR) is repeated in an inverted orientation internally (IR). This divides the unique region into long unique (U_L) and short unique (U_S) regions. The short region can invert giving two isomers of viral DNA. In group E genomes, such as in herpes simplex virus and human cytomegalovirus, one terminus contains N repeats of sequence A next to a longer sequence B. The other terminus has one directly repeated sequence A next to a sequence C. The terminal sequences are inserted in an inverted form internally (B' A'_N C'). Both the long and short regions can invert, forming four isomers. Repeat sequences have not been identified in group F genomes (adapted from Roizman, 2001c).

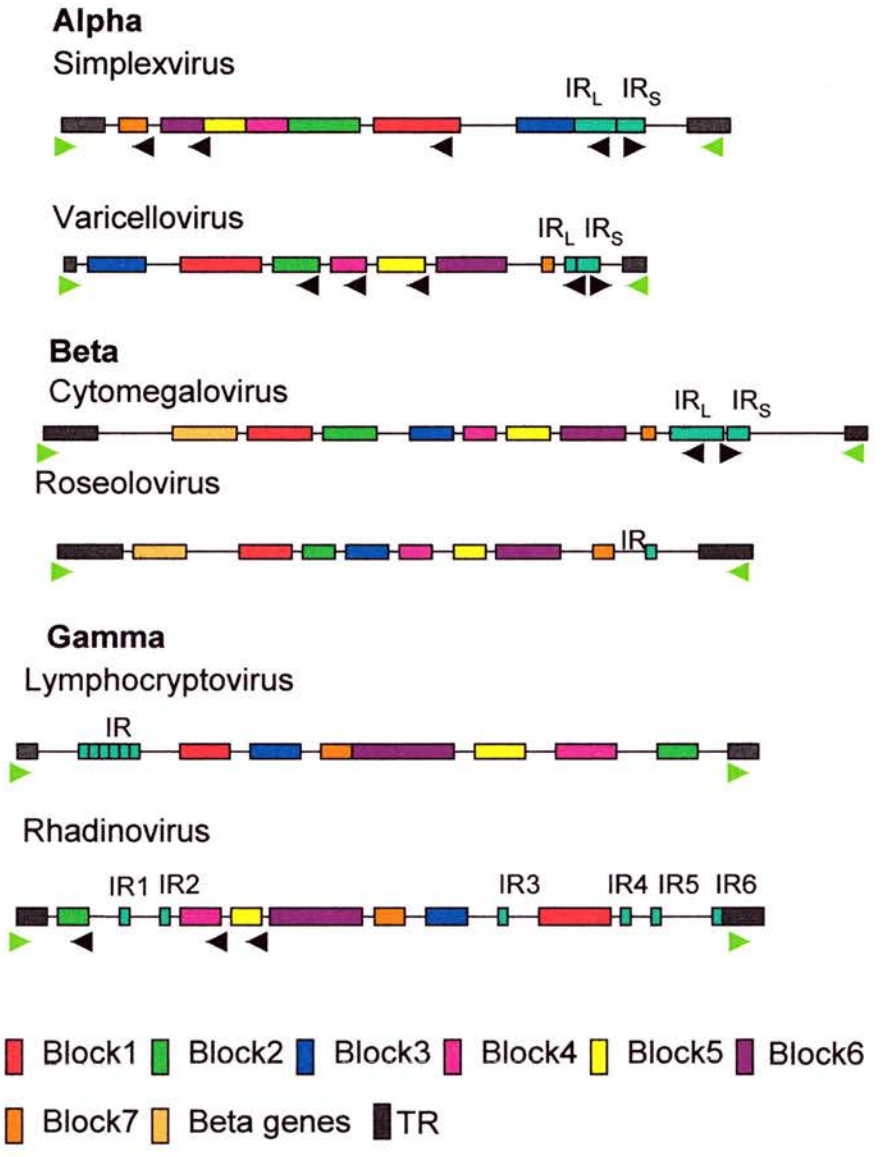
1.1.6 Herpesvirus Genes

The general features of herpesvirus genes are reviewed in Roizman, (2001c). Most herpesvirus genes contain a promoter and other regulatory sequences spanning 50 to 200bp upstream of a TATA box, a transcriptional initiation site 20 to 25bp downstream of the TATA box, a 5' non translated leader sequence of 30 to 300bp, a single major ORF, with a translation initiation codon that meets the host requirements for efficient initiation, 10 to 30bp of 3' non-translated sequence and a polyadenylation signal. Exceptions are found, e.g. HSV-1 $\gamma_1$34.5 gene has no TATA box and in some HSV late genes the promoter regulatory sequences may be located 3' to the TATA box. Gene overlaps are common and ORFs can be expressed that are situated entirely antisense to each other. Most genes are transcribed by RNA polymerase II, however EBV encodes a set of small non-polyadenylated RNAs or EBERs that are not translated and are transcribed by RNA polymerase III. Most herpesvirus genes are not spliced, however every herpesvirus encodes some spliced genes and it is thought that splicing might enable differential regulation of a gene at different parts of the virus life cycle.

1.1.7 Classification of Herpesviruses by Gene Relatedness and Arrangement

Herpesvirus genomes contain between 70 and 200 ORFs. Among the viruses of mammals and birds the genes are arranged into seven gene blocks. These gene blocks have different orders and orientations in different herpesvirus subfamilies (Figure 1.3), but genes within a block maintain order and transcriptional polarity. A subset of 26 genes are conserved among the alpha, beta and gammaherpesviruses, these genes are known as the herpesvirus core genes and include capsid proteins, tegument proteins and components involved in DNA replication. Some genes are conserved within subfamilies and some herpesviruses, especially the gammaherpesviruses, encode homologues of cellular genes. It is hypothesised that these genes might be acquired from the host. The cellular homologues are usually located towards the viral termini between gene blocks. The complete genome sequences for 26 herpesvirus species is now available and these sequences can be used to construct phylogenetic trees illustrating the genetic relationships between the

Figure 1.3 Conserved Gene Blocks of the Alpha, Beta and Gammaherpesviruses



The gammaherpesvirus genes are arranged into seven blocks which are colour coded as shown. The beta genes are found only in betaherpesviruses. Gene blocks inverted relative to the betaherpesviruses are marked with black arrowheads. Terminal repeats (TR) are colour coded as shown, green arrowheads show the direction of the terminal repeats. Internal repeats are marked IR. (Adapted from Roizman, 2001c).

herpesviruses (Davison, 2002). Virus sequence can be used along with criteria such as host range and biological properties to classify viruses. Sequence data has been used to classify viruses for which the complete sequence is not known, for example porcine lymphotropic herpesvirus-1 (PLHV-1)(Goltz *et al.*, 2002). The nucleotide sequence of a 200bp polymerase chain reaction product, amplified from the conserved herpesviral gene DNA polymerase gene by the use of degenerate primers, is sufficient to establish the identity as a herpesvirus and classify it into the appropriate subfamily. Sequences of genes such as glycoprotein B (gB), which is conserved throughout the herpesviruses, have been used for comparison of herpesviruses in order to classify them down to the genus level.

1.1.8 Herpesvirus Life Cycle and Replication

The life cycle of herpesviruses occurs in two different stages. The lytic stage involves viral replication, production of new herpesvirus virions and release from the cell, usually involving lysis of the infected cell. In the latent stage, the virus genome persists in the cell as an episome, with no production of new virions and expression of only a small subset of viral genes. The latent viral genome retains the ability to undergo reactivation, whereby the virus returns to the lytic stage of the life cycle and the full spectrum of viral genes is again expressed. It is thought that reactivation of HSV can be triggered by a number of stimuli such as stress, tissue damage, or immunosuppression, but the molecular mechanisms controlling latency and reactivation are not fully understood (Wagner & Bloom, 1997).

1.1.9 Herpesvirus Lytic Replication

Much of the work on lytic replication of herpesviruses has been done on herpes simplex virus and is reviewed in Roizman, (2001a) and Ward & Roizman, (1994). Lytic replication is described here as it occurs in herpes simplex virus (Figure 1.4). Since many replication genes are conserved between herpesviruses, the general principles are relevant to any herpesvirus.

To initiate infection the virus must attach to the cell surface. This involves the interaction of the viral envelope glycoproteins gC and gB with the glycosaminoglycan moieties of cell surface heparin sulphate. The next step involves

Figure 1.4 Lytic Replication of Herpes Simplex Virus

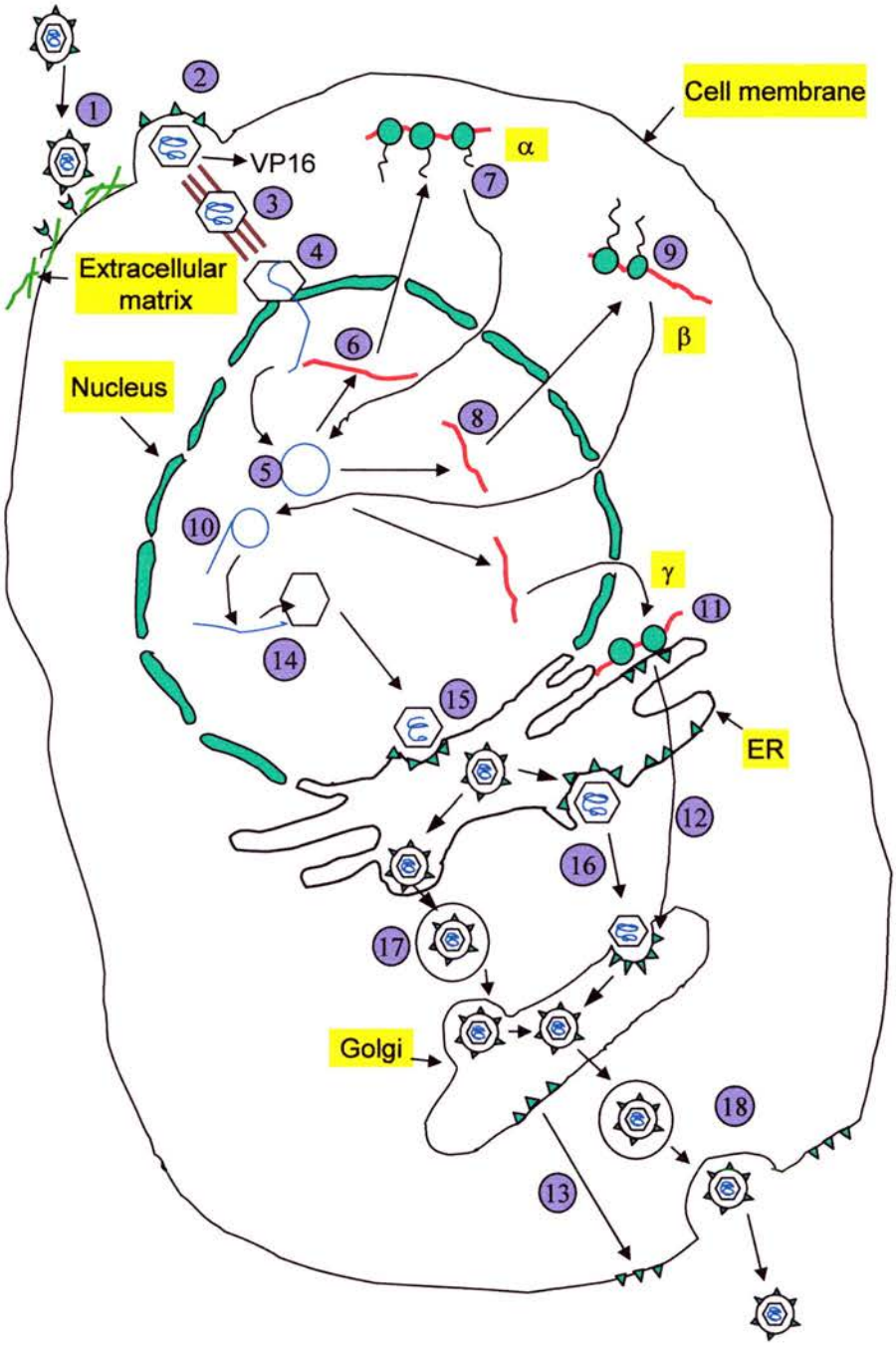


Figure 1.4 The diagram shows the lytic replication cycle of herpes simplex virus. To initiate infection, the virus attaches to the cell surface by interaction of the viral envelope glycoproteins gB and gC with cell surface heparin sulphate (1). Glycoprotein D then interacts with one of several cellular co-receptors. This is followed by fusion of the viral envelope with the host cell membrane (2).

After membrane fusion the viral capsid is released into the cytoplasm. Viral capsids attach to microtubules and are transported to the nucleus (3). Certain tegument proteins, e.g. VP16 which activates transcription of the viral genome, are also transported to the nucleus. The viral DNA is released from the capsid into the cell nucleus through the nuclear pores (4). Once in the nucleus the viral DNA circularises (5). VP16 interacts with the host transcriptional components to stimulate transcription of the immediate early or α genes (6).

The RNA transcripts are transported to the cytoplasm and translated (7). The α proteins are transported to the nucleus, where they activate transcription of the early or ß genes (8). Early gene transcripts are transported into the cytoplasm and translated (9). The ß proteins function in viral DNA replication in the nucleus (10). Late or γ genes encode virion structural proteins and those needed for virus assembly and particle egress. Some γ proteins are required for formation of the viral envelope and are synthesised on the rough ER (11). These membrane proteins are modified by glycosylation.

Precursor viral membrane proteins are thought to be located on the inner and outer nuclear membranes and on the membranes of the rough ER. These precursors are transported to the Golgi apparatus for further modification and processing (12). Mature glycoproteins are transported to the plasma membrane of the infected cell (13). Newly replicated DNA is packaged into capsids (14). DNA containing capsids bud from the inner nuclear membrane into the lumen of the ER (15), acquiring an envelope containing precursors of viral envelope proteins.

Two possible mechanisms have been suggested for transport of enveloped virus to the cell membrane. In the de-envelopment pathway (16), enveloped virus fuses with the ER membrane and is released into the cytoplasm. The capsid buds into a late Golgi component, where it acquires a membrane containing mature viral envelope proteins. The enveloped virus is then transported to the cell membrane for release by exocytosis. In the luminal pathway (17) enveloped virus is engulfed in a vesicle and delivered to the Golgi, where the envelope proteins are modified by Golgi enzymes. The enveloped virus is then transported to the cell membrane for release by exocytosis (18). (Adapted from Flint *et al.*, (2000)).

the interaction of the viral glycoprotein gD with one of several cellular co-receptors. The co-receptors include members of the tumour necrosis factor (TNF) receptor family and members of the immunoglobulin superfamily. It is also thought that 3-O-sulphated heparin sulphates may serve as receptors for viral entry (Shukla *et al.*, 1999). This is followed by fusion of the viral envelope with the host cell membrane, requiring gB, gD and a gH-gL heterodimer. After fusion the capsid is transported to the nuclear pore, whilst the tegument proteins are thought to modify cellular metabolism.

After entry into the cell nucleus the viral DNA circularises. In cells permissive for viral replication, entry of the virus genome into the nucleus is followed by a highly regulated cascade of viral gene expression. Host RNA polymerase II is responsible for transcription of all the viral genes (Costanzo *et al.*, 1977) and all viral proteins are synthesised in the cytoplasm. Immediate-early, or alpha genes are defined by their transcription in the presence of inhibitors of protein synthesis, such as cyclohexamide. The immediate early genes are distinct among different subfamilies, and regulate subsequent gene expression by transcriptional and post-transcriptional mechanisms. In HSV there are six alpha genes, which encode viral proteins designated as ICP0, ICP4, ICP22, ICP27, ICP47 and $U_s1.5$, whose transcription unit lies within the coding sequence of the gene encoding ICP22. The virion tegument protein VP16 stimulates the transcription of the alpha genes (Campbell *et al.*, 1984).

The early or beta genes encode the DNA replication complex and a variety of proteins and enzymes involved in modifying host cell metabolism, whilst the late or gamma genes encode the structural proteins of the virus. HSV infection inhibits host transcription, RNA splicing and transport, to change the cell from cellular to viral gene expression. Viral DNA synthesis occurs by a rolling circle mechanism, from one or more origins of replication (Garber *et al.*, 1993). Replication of HSV-1 DNA requires seven gene products, comprising an origin binding protein, a single stranded DNA binding protein, a DNA polymerase composed of two subunits and a helicase-primase complex composed of three gene products. Homologues of all but the origin binding protein have been identified in all three herpesvirus subfamilies. Replication of viral DNA results in concatemers of viral DNA, which are cleaved into

monomers. This process is linked to the packaging of DNA into preformed mature capsids within the nucleus (Deiss & Frenkel, 1986).

After packaging the virus matures and acquires infectivity by budding through the inner lamella of the nuclear membrane, however it is not certain how generation of the full enveloped particle is carried out (Roizman, 2001a). It is thought that the virus may become de-enveloped by fusion with the nuclear membrane and may then be re-enveloped in the Golgi apparatus. The alternative theory is that the virion buds through the outer nuclear membrane, with the envelope intact and then enters the Golgi already enveloped. Whilst in the Golgi, the oligosaccarides of the virion glycoproteins are processed by Golgi enzymes. The enveloped virions are transported through the Golgi to the cell surface in vesicles, where they are released from the cell. Cells productively infected with herpes viruses do not usually survive, as they suffer irreversible damage due to both viral replication and cellular responses to infection.

1.1.10 Latency

Every herpesvirus studied to date establishes latent infection in a specific cell type (Roizman, 2001c). The mechanisms of the establishment of latency vary from one virus to the next and the genes expressed during latency are not generally conserved between herpesviruses. Some viruses for example EBV, encode several proteins that are expressed during the maintenance of latency. In other herpesviruses for example HSV, gene expression during latency is more limited. Due to this variation, latency in herpesvirus will be discussed in the context of specific viruses.

1.2 Gammaherpesviruses

The gammaherpesviruses include pathogens of animals and man and are associated with lymphoproliferative diseases. EBV is the only human LCV and Kaposi's sarcoma-associated herpesvirus (KSHV) is the only human rhadinovirus. Lymphocryptoviruses have only been identified in primate species; many old world and new world primates have their own endemic LCV. In contrast, the rhadinoviruses are found in both primates and non-primate species. Gammaherpesvirus genomes include genes that are shared by most herpesviruses,

genes that are shared among gammaherpesviruses and genes conserved within genera. Many gammaherpesviruses encode homologues of cellular genes, these are located in the genome in the regions between blocks of conserved genes (Figure 1.4).

1.2.1 Epstein-Barr Virus

Epstein-Barr virus (EBV) was discovered in association with the malignancy Burkitt's Lymphoma, and therefore EBV became the first candidate human tumour virus discovered (reviewed in Kieff, 2001). EBV is also the prototype gammaherpesvirus. EBV is a ubiquitous infectious agent; over 90% of the world's population are infected. Primary infection usually occurs in early childhood and is followed by a lifelong carrier state. If primary infection is delayed however, it can be accompanied by the disease infectious mononucleosis (IM). EBV is also linked to the human cancer nasopharyngeal carcinoma (Wolf *et al.*, 1973) and is associated with post transplantation lymphoproliferative disease (Tanner & Alfieri, 2001), Hodgkin's disease and oral hairy leukoplakia.

1.2.1.1 The Structure of the EBV Genome

Due to the limited host range for EBV replication *in vitro* and the difficulty of obtaining large amounts of viral DNA for molecular biological investigation, EBV was the first herpesvirus whose genome fragments were cloned into *E. coli* (Arrand *et al.*, 1981) and was the first herpesvirus genome to be completely sequenced. The EBV genome was sequenced from a *Bam*HI library, therefore genes are often referred to specific *Bam*HI fragments. The EBV genome is a linear double stranded 172 kbp DNA molecule, with a base composition of 60% GC. The genome contains 0.5kbp reiterated terminal direct repeats and reiterated 3kbp internal direct repeats. These divide the genome into unique long and unique short regions. The unique long and unique short regions contain imperfect tandem DNA repeats, mostly within ORFs. The reiteration frequency of the EBV tandem repeats is variable during viral DNA replication, with the average number of repeats being equal to that of the parent genome. If cells are latently infected with episomal viral DNA, each EBV genome in the progeny infected cells will have the same number of terminal repeat units as the in the parent genome. This fact is used to determine if a group of latently infected

Figure 1.5 Comparison of the Genomic Arrangement of Gammaherpesviruses

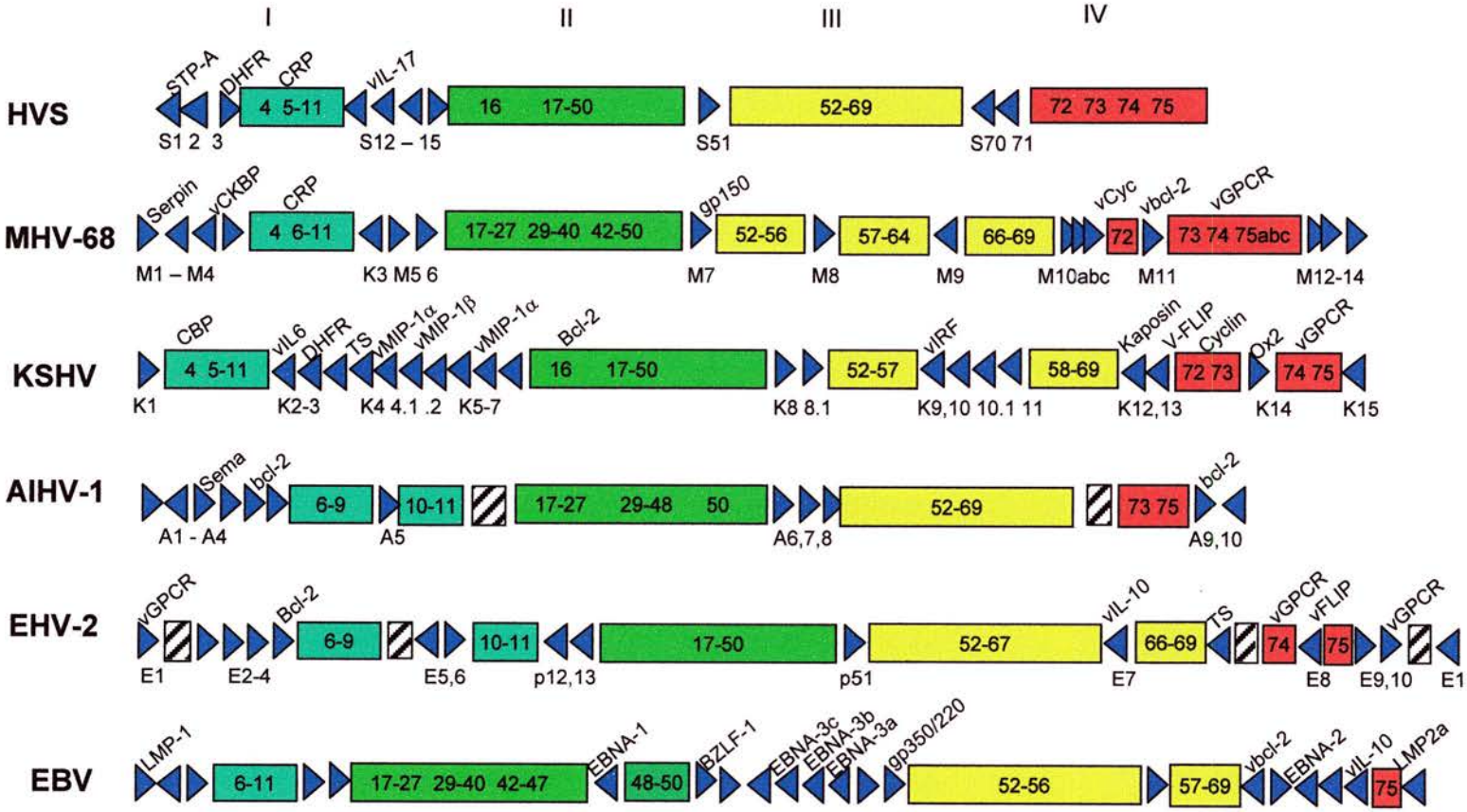


Figure 1.5 Comparison of the genomic arrangement of gammaherpesviruses. The genomes of herpesvirus saimiri (HVS), murine gammaherpesvirus-68 (MHV-68), Kaposi's sarcoma herpesvirus (KSHV), equine herpesvirus 2 (EHV-2), and Epstein-Barr virus (EBV) are shown. The EBV genome is shown inverted relative to its conventional orientation. The conserved gene blocks I (Turquoise), II (green), III (yellow) and IV (red). The numbers of open reading frames in the blocks are shown, and relate to the HVS open reading frame numbering system.

Interspersed between these blocks are ORFs which are unique to each family member; these areas contain homologues of cellular genes. Positional homologues to HVS ORFs are marked with the prefix "p". Gammaherpesvirus genes with no homologue in HVS are numbered separately: M, MHV-68 specific ORFs, K, KSHV specific ORFs, A, AlHV-1 specific ORFs, E, EHV-2 specific ORFs. Other abbreviations used are: STP – saimiri transforming protein, CRP – complement regulatory protein, DHFR – dihydrofolate reductase, vIL – viral interleukin, TS – thymidylate synthase, vFLIP – viral FLICE inhibitory protein, vMIP-1α/ß – viral macrophage inhibitory factor α/ß, vGPCR – viral G protein coupled receptor, sema – semaphorin, vIRF – viral interferon responsive factor. Non-coding regions in the genomes of AlHV-1 and EHV-1 are shown as diagonally striped blocks. (Adapted from Simas & Efstathiou, (1998))

cells arose as a result of clonal expansion from one progenitor cell, or from a number of cells (Raab-Traub & Flynn, 1986). Two EBV types exist, designated type 1 and type 2. Type 1 is more common in most populations; the main differences between the two types lie in the genes encoding the EBV nuclear antigens (EBNA).

The EBV genome has large areas of collinear homology at the amino acid level with other herpesviruses such as VZV and HSV. The relatively conserved domains encode EBV genes functioning in lytic infection. The genes expressed in latent EBV infection generally have no homology to other herpesviruses. It is thought that some may have arisen partly from cellular DNA, particularly EBNA-I, which contains repeat motifs found in host cell DNA (Heller *et al.*, 1985). The immediate early gene BZLF1 is closely related to the *jun/fos* family of transcriptional activators (Farrell *et al.*, 1989). EBV also has two bcl-2 homologues, encoded by the BHRF1 gene and the BALF1 gene. EBV also contains a homologue of the cellular gene IL-10 encoded by BCRF1. The BCRF1 gene shares 90% amino acid homology to human IL10 sequence (Moore *et al.*, 1990) (Hsu *et al.*, 1990).

1.2.1.2 Epstein-Barr Virus Replication

EBV can infect primary B-lymphocytes *in vitro*, initiating a latent infection in approximately 10% of cells. EBV infected B cells proliferate as immortalised or transformed latently infected LCLs. These cell lines have been used to identify and characterise the viral proteins expressed during this infection. Virus replication in these LCLs is minimal or undetectable. There is no *in vitro* system that is fully permissive for EBV replication, therefore studies of lytic infection have used semi-permissive LCL or Burkitt's lymphoma (BL) cell lines, where replication can be induced in a variable proportion of cells.

Lytic EBV infection is usually studied by inducing latently infected Burkitt's lymphoma cells to become permissive for lytic virus infection. This can be done in a number of ways. Treatment of cells with phorbol esters such as TPA results in protein kinase C activation, resulting in *jun-fos* interaction with Ap1 sites upstream of the immediate early genes of EBV (Baumann *et al.*, 1998). The Akata EBV infected cell line can be induced to 20 – 50% lytic infection by cross-linking surface immunoglobulin. This causes a cascade of second messengers, which leads to the mobilisation of calcium from intracellular stores and activation of protein kinase C.

The latent EBV genome is extensively methylated (Falk & Ernberg, 1993). Extensive methylation of lytic genes and their regulatory sequences is thought to help maintain latency by inhibiting lytic gene expression. Treatment of latently infected cells with drugs that reduce DNA methylation such as 5-aza-cytidine (Ben-Sasson & Klein, 1981), increases the frequency of spontaneous reactivation. Treatment of cells with sodium butyrate inhibits histone deacetylase and this relieves transcriptional repression resulting from condensed chromatin.

Entry of EBV into host B cells is initiated by interaction of the viral envelope glycoprotein gp350/220 with the complement protein C3d receptor CD21 (Fingeroth *et al.*, 1984). It has also been shown that a complex of three glycoproteins, the gH homologue gp85, the gL homologue gp25, and gp42, are required for penetration of the virus into host cells (Molesworth *et al.*, 2000). Gp42 binds HLA class II (Li *et al.*, 1997) and B cell lines which lack expression of HLA class II are not susceptible to EBV infection. The mechanisms of infection of epithelial cells with EBV are thought to be different from those of B cells. It is thought that a two part complex of gp85 and gp25 are involved in infection of epithelial cells with EBV (Molesworth *et al.*, 2000) (Maruo *et al.*, 2001), rather than the gp85/gp25/gp42 complex involved in infection of B cells. Some epithelial cells do express small amounts of CD21 and interaction of gp350 with CD21 has been shown to be of importance in infection of epithelial cells (Fingeroth *et al.*, 1999). Epithelial cells and B cells have been shown to be infected independently of gp350 and CD21 (Janz *et al.*, 2000) and it is thought that other viral and cellular molecules are involved in the binding of EBV to its target cells.

The immediate early gene products of EBV are BZLF1 or Zta, and BRLF1 or replication and transcription activator (Rta). The proteins encoded by these genes are key transactivators of a cascade of lytic EBV gene expression. BZLF1 and BRLF1 up regulate gene expression from early EBV gene promoters. Approximately 30 EBV genes have been classified as early genes. Many early genes are involved in viral DNA replication, such as the viral DNA polymerase (BALF5) and ribonuclease reductase (BORF2). The early genes BSMF1 and BMRF1 function as transcriptional activators that can transactivate the expression of other EBV early genes. The EBV bcl-2 homologues BHRF1 and BALF1 are both expressed in early lytic infection.

BHRF1 is thought to be important in preventing apoptotic cell death during lytic replication (Henderson *et al.*, 1993), whilst BALF1 acts to negatively regulate the antiapoptotic activity of BHRF1.

Many EBV late genes studied are glycoproteins and have potential importance in antibody-mediated immunity to virus infection. Knowledge of non-glycoprotein late genes is limited. One of the most abundant EBV late proteins is the gB homologue gp110, encoded by BALF4. This protein is found in large amounts on the inner nuclear membrane, however only small amounts are found in enveloped extracellular virus. The gp110 protein is both N and O glycosylated. Due to its distribution in the inner and outer nuclear membranes and cytoplasmic membranes surrounding virus, but not in Golgi or plasma membrane, gp110 is thought to have a role in initial envelopment of virus.

The late proteins gp85 and gp350/220 are found in the Golgi and on the plasma membrane of infected cells. The glycoprotein gp350/220 is encoded by BLLF1, which has a small region of distant homology to HSV-1 gC (Beisel *et al.*, 1985). The glycoproteins Gp350 and gp220 are encoded by the same gene and the mRNA encoding gp220 is spliced in frame. Gp350/220 is the major external virus glycoprotein, which mediates binding to the CD21 B lymphocyte receptor. Gp85 is found in smaller amounts and is important in fusion of the virus envelope and the host cell membrane. The glycoprotein gp85 has significant collinear homology with HSV gH (Heineman *et al.*, 1988) and complexes with the BKRF2 protein, which is homologous to HSV gL. This complex is homologous to the gH/gL complex with is found on the envelope of HSV.

Although gp350/220 is the most abundant viral protein in the lytically infected cell membrane and virus envelope, only small amounts accumulate in the lytically infected cell nuclear membrane. It is thought that the virus acquires the initial envelope as it buds through the nuclear membrane, de-envelopment then occurs in cytoplasmic vesicles, and nucleocapsids are released into the cytoplasm. Re-envelopment then occurs at the plasma membrane, where the virus receives a definitive envelope rich in gp350/220 and the gp85/BKRF2 complex.

The EBV IL-10 homologue BCRF1 is also expressed in early and late lytic infection. Cellular IL-10 has anti-inflammatory activities mediated by inhibition of

macrophage and monocyte synthesis of pro-inflammatory cytokines such as TNFα, IFNγ, IL-12 and IL-6 (Fiorentino *et al.*, 1991). IL-10 inhibits MHC class II expression on monocytes and inhibits proliferation of CD4+ T cells, but induces proliferation and cytotoxic activity of CD8+ T cells (Groux *et al.*, 1998). Cellular IL-10 can also induce proliferation of thymocytes and mast cells and has been shown to enhance the survival and differentiation of B cells (Moore *et al.*, 2001). The EBV viral IL-10 has 90% amino acid identity with human IL-10 (Hsu *et al.*, 1990). The main differences lie in the N terminal twenty amino acids. Despite the similarity with cellular IL-10, the EBV IL-10 only retains a subset of the activity of cellular IL-10. The EBV IL-10 retains the immunosuppressive activities such as inhibition of antigen presentation by monocytes (de Waal Malefyt *et al.*, 1991), inhibition of T cell proliferation in response to antigen and inhibition of interferon γ production by activated lymphoid cells (Hsu *et al.*, 1990). The immunostimulatory activities are however, not retained. The ability to reduce inflammation and antigen presentation is thought to aid viral persistence in the host by reducing the immune response to infection.

1.2.1.3 EBV Latency

EBV latency is complex and has been studied mainly using *in vitro* cell lines. Using these cell lines, latency has been classified into three types. The transformation process in these cells is associated with an ordered sequence of virus gene expression known as latency III. The features of latency III are;

- Expression of the EBV small non polyadenylated RNAs or EBERs
- Expression of the BamHI A RNAs, a family of spliced polyadenylated transcripts whose protein products are not well characterised.
- Six differentially spliced transcripts, expressed either from the BamHI W promoter or Wp, or more commonly from the adjacent BamHI C promoter or Cp. The transcripts encode the viral nuclear antigens EBNAs 1, 2, 3A, 3B, 3C and LP.
- Three spliced BamHI N RNAs encoding the latent membrane proteins LMP1, LMP2A, and LMP2B.

The other two forms of EBV latency, latency I and latency II, display a more restricted pattern of EBV gene expression. These two forms of latency are thought to represent two ends of a spectrum of viral gene expression, latency I and latency II differ only with respect to the activity of the LMP promoters.

The features of latency I are;

- Expression of the EBERs
- Expression of the BamHIA RNAs.
- Silencing of the Cp/Wp promoters and activation of a downstream promoter Qp, which initiates transcription of an EBNA I RNA with a unique splice structure compared with expression from Cp/Wp.
- Silencing of the LMP promoters, making EBNA I the only virus-encoded protein expressed in type I latency.

The features of Latency II are:

- Expression of the EBERs,
- Expression of the BamHI A RNAs
- Expression of EBNA I from the Qp promoter, as seen in latency I.
- Activation of one or more LMP promoters, leading to expression of the LMPI and one or more of LMP2A and LMP2B mRNAs at levels varying from borderline to easily detectable.

EBV infected cells displaying some of the features of *in vitro* latency have been identified *in vivo*. Latency I was first identified in Burkitt's lymphoma cells, and latency II is found in nasopharyngeal carcinomas (Young *et al.*, 1988). RT-PCR analysis of viral transcription of freshly isolated B cells from patients with infectious mononucleosis has detected many of the RNAs characteristic of a latency III infection (Tierney *et al.*, 1994). Latency *in vivo* however remains difficult to study because the cells isolated for analysis may differ with respect to viral gene expression, therefore the results obtained will reflect a population of cells rather than individual EBV infected cells.

1.2.1.4 Functions of EBV Latency Associated Genes

The viral gene products expressed in latency maintain the latent infection and cause previously resting B cells to continuously proliferate. EBV infected proliferating lymphocytes display an activated phenotype similar to that seen in B lymphocytes proliferating in response to antigen, mitogen or IL-4.

EBNAs

The promoter Wp promotes the transcription of the first EBV RNAs, which are differentially spliced to encode EBNA-LP and EBNA-2. These two proteins up regulate transcription from viral and cellular promoters, including Wp and Cp (Harada & Kieff, 1997). EBNA-2 is required for *in vitro* growth transformation of EBV (Cohen & Kieff, 1991). The EBNA-2 protein up regulates expression of the cellular genes CD23, CD21, *c-fgr* and *c-myc* and *bcl-2* and the viral genes LMP1, LMP2, and EBNAs (Wang *et al.*, 1990). EBNA-1 is present in all EBV infected cells, regardless of the state of infection. It is essential for maintenance of viral episomes in infection, as it mediates binding of EBV to chromosomes via the oriP or latent origin of replication. The EBNA-1 gene contains a glycine and alanine (Gly-Ala) repeat domain. This domain is thought to be involved in the inhibition of presentation of EBNA-1 epitopes by interference with the antigen processing pathways (Levitskaya *et al.*, 1997). This is thought to prevent a CTL response to EBV infected cells expressing EBNA-1.

The EBNA 3 genes are tandemly placed in the genome. EBNA-3A and 3C are thought to be important for B lymphocyte growth transformation, however EBNA-3B is not essential for any aspect of lymphocyte infection *in vitro* (Tomkinson & Kieff, 1992). EBNA-LP is also thought to be important in host cell transformation, as cells infected with EBNA-LP mutants have a reduced transformation phenotype (Mannick *et al.*, 1991).

LMP1

LMP1 is a membrane protein and has been shown to be essential for B lymphocyte growth transformation by EBV (Kaye *et al.*, 1993). LMP1 also has transforming effects on non-lymphoid cells such as rodent fibroblasts (Wang *et al.*, 1985). In B

lymphocytes, LMP1 induces many of the phenotypic effects of EBV infection (Wang *et al.*, 1988), including induced expression of activation markers such as CD23, CD39 and CD40, adhesion molecules such as LFA-1 and ICAM-1 and LFA-3, the cell skeletal protein vimentin, HLA class II and the anti apoptotic genes *bcl-2* and A20. It has been shown a 200 amino acid carboxy terminal cytoplasmic domain of LMP1 is important for the growth transforming properties of LMP1 (Kaye *et al.*, 1995). LMP1 acts as a constitutively active receptor. The first 44 amino acids of the carboxy terminus interact with TNF associated cell cytoplasmic factors hTRAF3 and hTRAF1, initiating a signalling pathway which results in activation of the transcription factor NFκB.

LMP2

The LMP2 gene is expressed from a spliced mRNA that contains exons located at both ends of the EBV genome. LMP2 can thus only be transcribed in latency when the genome is circularised. Two forms of LMP2 are expressed; LMP2A and LMP2B. These forms differ only in the first exons; the remaining exons are shared by both. LMP2A has been shown to interfere with membrane signal transduction through phosphorylation of its hydrophobic amino terminal domain and binding of the cellular non-receptor tyrosine kinases Fyn and Lyn (Miller *et al.*, 1994). This binding blocks subsequent signal transduction from the immunoglobulin receptor and inhibits B cell activation that would activate the lytic cycle.

1.2.1.5 Persistence of the EBV Genome During Latency

The EBV genome persists in latently infected cells as a circular episome. Stable latently infected EBV cell lines contain multiple copies of EBV episomes (Adams & Lindahl, 1975) and most LCLs have about 10 copies of the EBV genome per cell. The Burkitt's lymphoma cell line Raji has 50 episomes per cell and has stably maintained this number for over 30 years in culture (Adams, 1987). The initial amplification of circular EBV DNA is at the start of latent infection and requires S phase DNA synthesis, as prior to S phase each infected cell only has one episome (Arvanitakis *et al.*, 1995). EBV DNA can also integrate into chromosomal DNA and both integrated and episomal DNA has been identified in some cell lines. Integration occurs when BL cell lines are infected with EBV and extensively passaged, however

it is not a regular feature of EBV infection. In latent infection, the EBV genome undergoes extensive methylation and is associated with chromosomal proteins. Regulatory domains involved in maintaining latent infection are undermethylated in latently infected cells relative to genes that are expressed in lytic infection (Falk & Ernberg, 1993).

1.2.1.6 Immune Response to EBV

Most of the information about antibody responses to EBV comes from the study of infectious mononucleosis (IM) patients (reviewed in Rickinson, 2001). By the onset of clinical symptoms, IM patients have IgM antibodies to the viral capsid antigen (VCA) (composed of BCLF1, BFRF3, BLRF2 and gp110) and a rising titre of IgG antibody to VCA and early antigen (EA). EA is composed of a mixture of immediate-early and early proteins including BZLF1, BALF2 and BHRF1. Virus neutralising IgM and IgG antibodies develop to the major neutralisation target antigen gp350, with IgG antibodies rising relatively late in disease. Antibodies to gp350 also have the capacity to mediate antibody dependant cellular cytotoxicity against cells in the late phase of virus replication (Pearson *et al.*, 1979). IgG Antibodies to EBNA-1 develop late in disease; healthy virus carriers maintain IgG antibodies to VCA, gp350 and EBNA-1.

The virus neutralising antibodies are not sufficient to protect against infection although they may prevent virus spread. It is thought that the cell mediated response plays a more important role in protection against EBV, as illustrated by the incidence of EBV associated lymphoproliferative disease in T cell immunosuppressed patients (Nalesnik, 1998), (Beral *et al.*, 1991). An EBV specific cytotoxic T lymphocyte cell (CTL) response occurs in IM patients (reviewed in Rickinson & Moss, 1997) and HLA class I restricted T cells can be detected specific to both latent and early lytic cycle antigens. The initial expansion of latently infected growth-transformed B cells seen in IM patients is eventually brought under control and it is thought that this is brought about by the virus specific CTL response.

1.2.1.7 Viral Persistence *In Vivo*

It is thought that the reservoir of EBV infection is contained in the B cell pool, as bone marrow transplant patients receiving irradiation to destroy their lymphoid system, but not other possible sites of persistence such as mucosal epithelium, were cleared of their EBV infection (Gratama *et al.*, 1988). Virus infected B lymphocytes are thought to differentiate into memory B cells. EBV is thought to persist in the memory B cell pool, exhibiting a restricted pattern of viral expression in order to avoid CTL surveillance (Laichalk *et al.*, 2002), (Babcock *et al.*, 2000). Little is known about how the pool of resting EBV infected B cells is maintained, however latency type III cells must be continually generated, as antibody levels to type III proteins are maintained at high levels for life.

1.2.1.8 Burkitt's Lymphoma

Burkitt's lymphoma (BL) is a B cell tumour originally identified in parts of Africa and Papua New Guinea (Burkitt, 1963), but is now known to be found worldwide in sporadic and endemic forms. BL is a disease of children and is rarely found in patients over the age of 14 years. The endemic form of Burkitt's lymphoma occurs in areas with high malaria infection, 97% of endemic BL tumours carry the EBV genome, whilst only 12 – 25% of sporadic BL tumours are EBV positive (Ziegler *et al.*, 1976). The 3% of EBV negative BLs occurring in endemic areas are thought to be sporadic cases of BL.

Tumours are found in a variety of sites but are most commonly found on the mandible or maxilla. In the absence of treatment, BL tumours grow quickly and death ensues within a few months. Chromosomal translocations are seen in BL cells of both the sporadic and endemic form, in which the c-*myc* oncogene on chromosome 8 is brought under the influence of an immunoglobulin gene promoter on chromosome 14, 22 or 2. The deregulation of the c-*myc* gene is thought to be responsible for the malignant change in the affected cell and the rapid outgrowth of a clone of tumour cells. The role of EBV and malaria infection in the pathogenesis is not clear, however it is thought to be a complex multistep process (Kieff, 2001). It has been suggested that rapid proliferation of EBV infected cells occurs in the presence of poor immunological control brought about by endemic malaria. This

rapid proliferation is thought to increase the chances of a chromosomal translocation occurring (Klein, 1987).

The Burkitt's lymphoma cell phenotype as determined by tumour biopsies, is that of a non-activated B cell. Viral gene expression in these cells is a restricted type I pattern with expression only of EBNA-1. In some tumours, however, a small number of cells reactivate expression of additional latent or lytic cycle genes (Niedobitek *et al.*, 1995).

1.2.1.9 Infectious Mononucleosis (IM)

Infectious mononucleosis occurs as a result of delayed primary infection with EBV. It is seen in teenagers and young adults of western societies and is sometimes known as "the kissing disease" (Rickinson, 2001). Symptoms seen are sore throat, fever, sweating, anorexia, headache and malaise. General lymphadenopathy occurs and is particularly marked in the pharyngeal region. Splenomegaly and hepatomegaly are also found. IM can sometimes resolve in days, but often continues for 1-2 weeks and is followed by a period of lethargy before complete recovery. One of the key features of IM is the presence of large numbers of "atypical mononuclear" cells in the blood. These are predominantly CD8+ T lymphoblasts although some CD4+ cells and NK like cells are found. There is expansion of a specific subset of CD8+ T cells with a Vß T cell receptor, which are thought to be proliferating in response to antigen (Callan *et al.*, 1996). The CD8+ T cells are specific for both latent and lytic EBV antigens (Callan *et al.*, 1998).

IM is thought to be an immunopathological disease whose symptoms are caused by pro-inflammatory cytokines such as IL-1, IFN-γ, and TNFα produced at high levels by the reactive T cells seen infiltrating all tissues of the body (Anagnostopoulos *et al.*, 1995). It is not known why EBV causes IM in adolescence, although it has been suggested that the cause could be the larger infectious dose of virus transmitted than is found in childhood.

1.2.1.10 Nasopharyngeal Carcinoma

Nasopharyngeal carcinoma (NPC) is a malignancy that develops in the squamus epithelium of the nasopharynx (Raab-Traub, 1993). The disease is the major cause of

death from cancer in southern China and also develops at high incidence in Mediterranean Africa and in Eskimo populations. The tumour is found in two types, 70% are the undifferentiated type and the rest show squamus differentiation.

EBV is detected in all undifferentiated NPC (Niedobitek *et al.*, 1991). Within each tumour the EBV is clonal, suggesting the tumour arose from proliferation of a single EBV infected cell (Raab-Traub & Flynn, 1986). Viral gene expression within NPC cells follows a latency type II pattern of gene expression and EBNA-1 and LMP1 expression is detected (Young *et al.*, 1988). In addition to EBV infection, there are other factors associated with development of NPC. It has been shown that Chinese populations have a genetic predisposition to NPC (Chan, 1983). Environmental factors also play a part; some traditional herbal remedies taken as snuff have been found to contain tumour promoting phorbol esters (Hirayama & Ito, 1981) and traditional salt fish dishes contain carcinogenic nitrosamines (Ho *et al.*, 1978). Phorbol esters are activators of EBV replication as well as having tumour promoting effects, therefore a higher number of EBV infected cells will be available to progress to malignancy, possibly through a combination of the effects of EBV LMP-I, with nitrosamine carcinogenicity and the tumour promoting effects of phorbol esters.

1.2.1.11 Lymphomas in Immunosuppressed Patients

The lifelong infection with EBV is usually controlled by cell-mediated immunity. The importance of control of EBV by the immune system is illustrated by the incidence of malignancies in immunosuppressed patients, for example, post transplantation, or suffering from acquired immunodeficiency syndrome (AIDS).

1.2.1.12 Post Transplantation Lymphoproliferative Disease (PTLD)

EBV is widely recognised as being associated with the development of lymphomas in transplant patients (Nalesnik, 1998). The intensity of T cell suppressive therapy given after transplantation is a major influence on the incidence of disease. After renal and liver transplants the incidence is 1 – 2%, however after heart and lung transplants the incidence is 3 – 8%, reflecting the higher amounts of immunosuppression given to these patients. PTLD is also seen after bone marrow

transplants (Nalesnik, 1998), especially when donor T cells are depleted from the marrow graft. Another major influence on the development of PTLD is EBV status. A higher incidence of PTLD is seen in EBV seronegative patients (Ho *et al.*, 1985), and therefore children are at a particularly high risk.

PTLD malignancies are found as a number of different types and 90% of the lesions carry EBV. The tumour cells express EBNA-1, EBNA-2 and LMP1, consistent with them being EBV transformed LCL-like foci proliferating in the absence of CTL surveillance (Young *et al.*, 1989). Many PTLD lesions, especially early lesions, regress when immunosuppression is reduced. This is thought to be due to the re-emergence of EBV specific cytotoxic T cells (Khatri *et al.*, 1999). Reduction of immunosuppression, however, carries with it the risk of rejection of the transplanted organ. Cases of PTLD have also been treated successfully by adoptive immunotherapy (Haque *et al.*, 2002). In this approach, CTL lines specific for EBV were established from healthy blood donors. The best available HLA matched CTLs were used to treat EBV positive PTLD in patients who has failed to respond to other forms of treatment.

1.2.1.13 Acquired Immunodeficiency Syndrome Lymphomas

HIV infected individuals are at particularly high risk of developing B cell lymphomas. Approximately half of lymphomas seen in AIDS patients are EBV associated; however association varies between different tumour types (Gaidano *et al.*, 1998). A major type of AIDS lymphoma is similar to PTLD lesions and appears in end stage AIDS when the patient is profoundly immunosuppressed. EBV is present in 80% of lesions, EBV positive tumours are largely composed of LMP1 positive, EBNA-2 positive B lymphoblasts typical of an EBV driven B cell proliferation. Burkitt's lymphoma type tumours also arise at a higher incidence in AIDS patients, though at an earlier time in disease than PTLD like lesions. These tumours are EBV positive in 30 – 40% of cases and EBV expression in these lesions is more restricted, typical of latency I type of expression pattern (Hamilton-Dutoit *et al.*, 1993).

1.2.2 Kaposi's Sarcoma Herpesvirus (KSHV)

The human *rhadinovirus* KSHV or Human Herpesvirus-8 (HHV-8), was first identified in an AIDS related Kaposi's sarcoma (KS) biopsy, and was the second of the human gammaherpesviruses to be identified. KSHV is also associated with the malignancies primary effusion lymphoma (PEL) and Multicentric Castleman's Disease (MCD).

1.2.2.1 Kaposi's Sarcoma

Kaposi's sarcoma is a vascular tumour consisting of bundles of spindle cells, an inflammatory infiltrate and prominent angiogenesis (reviewed in Jenner & Boshoff, 2002). The characteristic spindle cells are thought to be the tumour cells. The majority express lymphatic endothelial cell markers, however some express proteins more characteristic of smooth muscle, macrophages and dendritic cells, therefore it is thought that the spindle cells may be derived from a precursor cell whose progeny gives rise to haematopoietic and endothelial cells.

Kaposi's sarcoma is found in a number of associations. Classic Kaposi's sarcoma is described as an indolent tumour of elderly Mediterranean men. In central Africa, endemic KS affects young children (Ziegler & Katongole-Mbidde, 1996), and the occurrence of KS is increased among transplant recipients and chemotherapy patients, especially those in high risk ethnic groups (Penn, 1979). With the onset of the AIDS epidemic, AIDS-KS is now the most common form of KS.

KSHV DNA has consistently been detected in all forms of KS and is found in the spindle cells of the lesions (Boshoff *et al.*, 1995). It is not known if KS spindle cell proliferation is caused by viral gene products or by external inflammatory cytokines induced by infection. In the early stages of the disease only a small proportion of spindle cells are KSHV positive, however in the later stages of the disease, 90% of spindle cells contain KSHV, suggesting the virus provides a growth advantage to infected cells. It is thought that KSHV is a polyclonal hyperplasia in the early stages, which develops into a true clonal malignancy as the disease progresses.

1.2.2.2 Primary Effusion Lymphoma (PEL)

Primary effusion lymphoma is a B cell lymphoma and occurs as effusions in visceral cavities. The majority of PEL patients are HIV positive with advanced immunosuppression and 80% of cases contain latent EBV. PEL is thought to originate from post-germinal centre B cells, due to the presence of hypermutated immunoglobulin genes and markers of late stage B cell differentiation in PEL cells (Matolcsy *et al.*, 1998) (Gaidano *et al.*, 1997). Much of the research on KSHV has been undertaken using latently infected cell lines established from PEL, for example BCP-1 (Boshoff *et al.*, 1998) and BC-3 (Arvanitakis *et al.*, 1996).

1.2.2.3 Multicentric Castleman's Disease (MCD)

MCD is a lymphoproliferative disorder characterised by lymphadenopathy, episodes of fever and splenic infiltration (Peterson & Frizzera, 1993). MCD is more common in HIV infected individuals, where it is often an aggressive disease. KSHV is present in almost all cases of MCD in AIDS patients, and in around half of cases in HIV negative patients. KSHV positive MCD cases form a distinct class of MCD, named plasmablastic MCD. In this form of MCD large plasmablastic cells, all containing KSHV are found. Unlike PEL cells, coinfection with EBV has not been found in MCD plasmablasts (Dupin *et al.*, 2000).

1.2.2.4 KSHV Genome

Initial study of KSHV infected cell lines was hampered by lack of a replicative tissue culture system for this virus (Renne *et al.*, 1996b). TPA treatment of the KSHV infected cell line BCBL-1 resulted in an increase in the number of lytically infected cells and the first visualisation of the KSHV virus by electron microscopy. The complete genome of KSHV was sequenced by mapping genomic phage and cosmid libraries from the PEL cell line BC-1 (Russo *et al.*, 1996). The genome consists of a 140.5 kb long unique coding region, flanked by approximately 800bp terminal repeat sequences with a high GC content. In the long unique region, 81 open reading frames have been identified, including 66 with sequence similarity to herpesvirus saimiri (HVS) ORFs. The ORFs of KSHV are arranged in the seven gene blocks, similar to other herpesviruses. It was initially thought that KSHV was most closely related to

the prototype gammaherpesvirus HVS. The sequencing of strains of rhesus rhadinoviruses (Searles *et al.*, 1999), (Alexander *et al.*, 2000) has revealed that these viruses are also closely related to KSHV.

The KSHV genome contains a large number of genes encoding homologues of host genes (Neipel *et al.*, 1997), including those involved in induction of cellular proliferation, such as v-cyclin (ORF 72), v-G protein-coupled receptor (ORF 74), v-interferon regulatory factor (K9). Genes involved in inhibition of apoptosis include v-bcl-2 (ORF 16) (Cheng *et al.*, 1997), fas-associated death domain (FADD) interleukin-1ß-converting enzyme (FLICE) inhibitory protein (v-FLIP) (ORF 71), and v-IL6. Homologues of the chemokines Macrophage inflammatory protein (MIP)-I, II and III are encoded by K6, K4, and K4.1. ORF 4 is a complement binding protein homologue, and K14 encodes a neural cell adhesion molecule (NCAM)-like protein (v-adh), which is homologous to the rat and human OX-2 membrane antigens. It is thought that these cellular homologues enable KSHV to control host cellular responses and avoid anti-viral responses (Jenner & Boshoff, 2002).

1.2.2.5 KSHV Latency

KSHV is latent in the majority of *in vitro* KSHV infected cell lines, existing in the nucleus as closed circular episomal DNA (Renne *et al.*, 1996a). It is thought that the latent viral DNA is replicated by the host cell DNA replication machinery during cell division. During mitosis, KSHV DNA is tethered by the TR sequences to histone H1 on host chromatin via the KSHV latent nuclear antigen (LANA), encoded by ORF 73 (Ballestas *et al.*, 1999). This is similar to the function of EBV EBNA-1. The cells infected by KSHV are thought to be pre-germinal centre B cells in the case of MCD, post germinal centre B cells in the case of PEL and endothelial cell precursors in the case of KS. These cell types all have a high proliferative potential, which is thought to help the virus propagate its genome within the host. The virus displays a restricted pattern of gene expression in the cells it infects (Zhong *et al.*, 1996). All infected cells so far have been shown to express the latent nuclear antigen (LANA), encoded by ORF 73, v-cyclin (ORF72) and v-FLIP (ORF 71). These genes are adjacent in the genome and are co-transcribed on two polycistronic RNAs, LT1 (LANA, v-cyclin, v-FLIP) and LT2 (v-cyclin/v-FLIP) (Talbot *et al.*, 1999).

LANA has been shown to have a number of functions, as well as tethering viral episomes to host chromosomes in a similar way to EBV EBNA-1. LANA has been shown to bind p53, repressing its transcriptional activity and its ability to induce apoptosis, (Friborg *et al.*, 1999) this therefore may function to promote viral oncogenesis by enhancing survival of infected cells. LANA has been shown to up regulate a number of promoter sequences, including telomerase and can also transactivate expression from LT1 and LT2, maintaining latent gene expression. In addition, LANA has been shown to interact with the cellular proteins mSin3A, SAP30 and CIR, components of a corepressor complex that recruits histone deactylases to promoters (Krithivas *et al.*, 2000). Deacetylation of histones is associated with repression of transcription, it has been suggested that LANA1 may act to repress ORF 50 expression, thus maintaining latency. LANA also binds to RING3, one of the five human homologues of *fsh* of *Drosophila* and is phosphorylated as a consequence of this interaction (Platt *et al.*, 1999). LANA causes RING3 to relocate to nuclear heterochromatin regions, where it is thought that LANA and RING3 might have a role in regulation of chromatin structure (Mattsson *et al.*, 2002).

The viral encoded v-cyclin is most closely related to cyclin D2. It can interact with cellular cyclin dependant kinase (CDK) 6 to mediate phosphorylation of the retinoblastoma protein, leading to entry of the cell into S phase. The function of the anti-apoptotic v-FLIP is thought to be to protect cells against CD95 and tumour necrosis factor receptor 1 (TNFR1) - mediated apoptosis and possibly therefore against an attack by cytotoxic T lymphocytes (CTLs) (Djerbi *et al.*, 1999). The v-FLIP is expressed from an internal ribosome entry site (Bieleski & Talbot, 2001), this enables the mRNA to bypass the inhibition of cap-dependant translation in G2/M phase cell cycle arrested cells and ensures expression of v-FLIP throughout the cell cycle.

1.2.2.6 KSHV Lytic Replication

The majority of the KSHV genome remains silent during latency. This is thought to be due to methylation of promoter sequences such as that of the ORF 50 gene, which is highly methylated in latent PEL cell lines (Chen *et al.*, 2001). Lytic replication can

be induced by treatment with either phorbol esters such as 12-O-tetradecanoyl phorbol-13-acetate (TPA), or with sodium butyrate. KSHV ORF 50 or Rta is necessary and sufficient to activate the lytic cycle (Gradoville *et al.*, 2000). KSHV gene expression has been analysed in the TPA stimulated PEL derived cell line BC-3 by the use of microarrays (Paulose-Murphy *et al.*, 2001) (Jenner *et al.*, 2001). The first group of genes to be expressed after induction are those thought to encode regulators of gene expression such as ORF 50, K8, ORF 57 and ORF45. These are followed by genes involved in replication of DNA such as DNA polymerase (ORF9). Structural genes and genes involved in virus maturation are expressed as late genes. The ORFK8.1 encodes a viral envelope glycoprotein which is expressed late in infection (Raab *et al.*, 1998). This position in the genome is comparable to that of the EBV open reading frames BZLF2 or BLLF1, encoding the glycoproteins gp42 and gp350/220. The fully glycosylated protein gp35-37 has been shown to be part of the viral envelope (Li *et al.*, 1999c) and the serum of KS patients contains antibodies specific for the K8.1 protein product. The envelope glycoprotein K8.1 has been shown to interact with cell surface heparin sulphate (Birkmann *et al.*, 2001) and is required for efficient infection by HHV-8. It is thought that K8.1 may have functions in viral attachment comparable to the HSV glycoprotein gC, which has weak homology with EBV gp350/220.

A small percentage of cells in KS, PEL and PEL cell lines express markers of lytic replication (Parravicini *et al.*, 2000), in contrast to MCD where more infected cells express lytic genes. The presence of lytic gene transcripts in PEL, MCD and KS suggests lytic gene expression may be important in the pathogenesis of these diseases. The finding that the KSHV from KS, PEL and MCD has demethylated ORF 50 promoters compared to that from PBMCs supports this theory (Chen *et al.*, 2001). Lytic gene expression has been analysed in KS lesions. Some of the genes expressed have been identified as possibly important in the pathogenesis of KS. These are ORFK1, which can activate immuno-receptor tyrosine kinase signalling (Lee *et al.*, 1998), v-IRF-1 (ORFK9), which interferes with interferon signalling pathways (Gao *et al.*, 1997), and v-GCR (ORF 74), which acts as a constitutively active CXC chemokine receptor and induces the secretion of vascular endothelial growth factor (Schulz, 2000). In addition, a few virally infected cells in KS lesions

express viral chemokine homologues, the anti apoptotic *bcl-2* homologue and the viral IL-6 homologue (Staskus *et al.*, 1999). Viral IL-6 is also expressed in lytically infected cells in MCD and in PEL lesions and is thought to contribute to B cell growth in these lesions.

1.3. Animal Gammaherpesviruses

Animal gammaherpesviruses are of interest both in their own right as causes of disease, and as models to illustrate the variability and similarity of genome arrangements in gammaherpesviruses and to illustrate the differences in disease causing mechanisms.

1.3.1 Herpesvirus Saimiri

Herpesvirus saimiri (HVS) is the prototype *rhadinovirus*. The natural host of HVS is the squirrel monkey (*Saimiri sciureus*) in which it produces no apparent disease. The virus causes T cell lymphomas in new world primates other than its natural host and in experimental rabbits. Lymphoblastoid cell lines can be established from tumour tissues of marmosets and owl monkeys experimentally infected with HVS (Johnson & Jondal, 1981). These cell lines initially produce virus in 1 – 10% of cells (Schirm *et al.*, 1984), however after long term culture the ability to produce virus is lost. When the cells convert to non-producer status the virus persists as episomal DNA (Werner *et al.*, 1977). The cells in the cultures are T lymphocytes with the properties of natural killer cells. HVS causes malignant lymphomas in New Zealand white rabbits and lymphoblastoid cell lines can be propagated from the lymphomas (Ablashi *et al.*, 1985). A T cell line that grew in vitro in the presence of IL-2 was propagated from the spleen of a rabbit inoculated with HVS. Expression of HVS early antigens could be induced by addition of n-butyrate or TPA, however late antigens could not be induced and infectious virus could not be rescued from the cell line. On further analysis HVS DNA present in the cell line was found to contain a deletion in the unique region of the genome.

HVS can also transform human T lymphocytes to continuous IL-2 dependant growth (Mittrucker *et al.*, 1992). The transformed human T cells have the phenotype of activated mature T cells and possess the functional capabilities of normal T

lymphocytes in terms of proliferative response, lymphokine secretion and induction of IL-2 receptors. It is thought that the HVS transformed T cells grow via an autocrine mechanism in response to activation signals given by their CD2 molecules during mutual cell to cell contact.

The genome of herpesvirus saimiri has been completely sequenced (Albrecht *et al.*, 1992a) and consists of a 113kb low GC (34.5%) content unique region, flanked by about 35 non-coding high GC (70.8%) repeats of 1.4kb. The genomes of HVS and EBV are predominantly collinear and homologous genes are in approximately equivalent locations and relative orientations. At the left hand end of the L-DNA are seven U-RNA genes termed the herpesvirus saimiri U-RNAs (HSURs), including promoters for cellular U-RNAs. The HSURs are only expressed in productively infected cells and it is thought that these may contribute to cell transformation by degradation of cellular mRNAs involved in T cell growth regulation (Myer *et al.*, 1992). HVS contains two copies of the putative viral formylglycineamide ribotide amino transferase (v-FGARAT) at ORF 3 and ORF 75 in opposite orientations. FGARAT is a cellular enzyme involved in purine synthesis (Barnes *et al.*, 1994). A number of other gammaherpesviruses such as BoHV-4 (Zimmermann *et al.*, 2001) and AlHV-1 (Ensser *et al.*, 1997) encode two FGARAT ORFs in this same arrangement at opposite ends of the genome.

Sequence divergence of HVS isolates is found at the left hand end of the unique L-DNA and is the basis of classification of HVS into subgroups A, B and C (Medveczky *et al.*, 1984). Variation in this region is correlated with differences in the ability of the viruses to immortalise T lymphocytes *in vitro* and to produce lymphoma in non-human primates. Viruses of both subgroups A and C immortalise common marmoset T lymphocytes to IL-2 independent proliferation (Szomolanyi *et al.*, 1987). Subgroup C strains also immortalise rabbit and rhesus monkey lymphocytes and can produce lymphoma in rhesus monkeys, as well as in New World primates. The sequence variation is in open reading frames at the left hand end of the genome, which are essential for herpesvirus saimiri oncogenicity (Duboise *et al.*, 1998).

HVS subgroup A contains a single ORF, saimiri transforming protein A (STP-A) (Jung *et al.*, 1991), whereas sub group C contains two genes at this position,

STP-C, a divergent form of the STP gene and the tyrosine kinase interacting protein Tip. The protein Tip has been shown to physically interact with the protein tyrosine kinase Lck in HVS transformed cells (Jung *et al.*, 1995) inducing a constitutive action of Lck. It is thought that constitutive activation of Lck by Tip positively regulates the phosphorylation and activation of the signal inducer and activator of transcription (STAT) 3 protein, inducing transcription of genes involved in T cell growth (Isakov & Biesinger, 2000). STP-A interacts with a cellular src kinase (Lee *et al.*, 1997) whereas STP-C interacts with cellular ras (Jung & Desrosiers, 1995).

Other genes possibly contributing to stimulation of T cell proliferation by herpesvirus saimiri include ORF72, a cyclin homologue (Nicholas *et al.*, 1992) and ORF 74, a G protein coupled receptor homologue with homology to the interleukin-8 receptor (Ahuja & Murphy, 1993). In addition to the genes required for stimulation of cell proliferation, herpesvirus saimiri also encodes two potential inhibitors of apoptosis. ORF 16 encodes a functional homologue of the *bcl-2* oncogene (Nava *et al.*, 1997) in common with EBV and KSHV. ORF 71 encodes a viral FLICE inhibitory protein (v-FLIP). This gene has been found to prevent apoptosis, but is dispensable for viral transformation of T cells *in vitro* and for pathogenicity in cottontop tamarins *in vivo* (Glykofrydes *et al.*, 2000). Two other HVS genes encoding cellular homologues are ORF4 (Albrecht & Fleckenstein, 1992) and ORF 15 (Albrecht *et al.*, 1992b). These ORFs encode glycoproteins related to complement control proteins, which down regulate complement activation.

1.3.2 Murine Gammaherpesvirus-68 (MHV-68)

MHV-68 was first isolated from a bank vole in Slovakia (Blaskovic *et al.*, 1980) and is used as a small animal model for the study of gammaherpesvirus pathogenesis. In contrast to many other gammaherpesviruses, MHV-68 will undergo productive replication in tissue culture, also the availability of transgenic "gene knockout" mice enables manipulation of the host immune system. The *in vivo* function of viral genes can also be studied by production of recombinant viruses for infection of mice.

Mice inoculated intranasally with MHV-68 establish productive infection in the lung in alveolar epithelial cells, causing an interstitial and peri-bronchiolar pneumonia (Sunil-Chandra *et al.*, 1992a). The virus then spreads via lymph nodes to

the spleen, where it establishes latency in germinal centre B cells (Sunil-Chandra *et al.*, 1992b). MHV-68 has also been shown to establish latency in macrophages and dendritic cells (Flano *et al.*, 2000). The virus has also been shown to persist in the latent form in lung epithelial cells (Stewart *et al.*, 1998). Establishment of latency in the spleen is associated with splenomegaly and a mononucleosis similar to infectious mononucleosis found in humans after primary infection with EBV (Tripp *et al.*, 1997). MHV-68 infection leads to the development of lymphomas in mice (Sunil-Chandra *et al.*, 1994). A mouse B cell lymphoma was used to propagate the MHV-68 infected B cell line S11 (Usherwood *et al.*, 1996). This cell line carries MHV-68 predominantly in the latent form and is considered to be analogous to EBV positive Burkitt's lymphoma cell lines and KSHV positive PEL cell lines. The S11 cell line has been useful for studying MHV-68 latency *in vitro*.

The genome of MHV-68 has been completely sequenced (Virgin *et al.*, 1997), and displays the layout typical of a gammaherpesvirus with blocks of conserved genes separated by virus specific genes. In common with other gammaherpesviruses, MHV-68 has a number of genes of cellular origin and also contains homologues of other viral genes which may be important in pathogenesis. ORF4 has homology with various complement control proteins. MHV-68 encodes a D-type cyclin homologue, in common with HVS and KSHV and an IL-8 homologue (v-GPCR) as in KSHV. M11 has homology with *bcl*-2 and has been shown to be functional (Roy *et al.*, 2000). During lytic infection there is a conventional cascade of gene expression, immediate early genes such as ORF 50 and ORF 73 (LANA), early genes such as TK and late genes such as gp150 have been identified.

The glycoprotein gp150 is equivalent to gp350/220 of EBV (Stewart *et al.*, 1996). A virus glycoprotein has also been identified in this genomic position in all gammaherpesviruses studied (bovine herpesvirus 4, HVS, equine herpesvirus-2, KSHV). In common with gp350/220, antibodies to this protein neutralise virus infectivity in the absence of complement. The glycoprotein gp150 is translated from an unspliced open reading frame in contrast to that of gp350/220, where splicing occurs to generate gp220. Gp150 is glycosylated and in infected cells is localised to the nuclear margins, in the cytoplasm and on the cell surface. Gp150 is also located on the surface of the virion. At the left hand end of the viral genome is a group of

genes unique to MHV-68; many of these genes are associated with MHV-68 latency. These include four protein-coding genes named M1 to M4 and eight tRNA-like sequences. The M3 gene encodes a secreted chemokine binding protein that is expressed during acute infection and persistence (Bridgeman *et al.*, 2001). M1 has been shown to be non-essential for lytic replication *in vitro* and for latency *in vivo* (Clambey *et al.*, 2000). M2 is expressed during latency *in vitro* and *in vivo* (Husain *et al.*, 1999). The tRNA-like genes are expressed during productive infection *in vivo* and are expressed in splenic germinal centres in latently infected mice (Bowden *et al.*, 1997). The tRNAs therefore are considered to be a marker for latent infection, however their function is unknown. Study of the virus MHV-76, a deletion mutant of MHV-68 lacking the M genes and all of the viral tRNA like genes emphasised this region of the genome as important for viral pathogenesis (Macrae *et al.*, 2001), as this virus was less pathogenic *in vivo* and infected mice had lower levels of latency in the spleen.

1.4 Gammaherpesviruses of Veterinary Relevance

1.4.1 Equine Herpesvirus-2 (EHV-2)

EHV-2 is one of the five recognised herpesviruses for which the horse is the natural host. The biological properties of EHV-2 and 5 initially placed them as cytomegaloviruses, however sequence analysis of random fragments of their genomes led them to be re-classified as gammaherpesviruses. EHV-2 is widespread and can be isolated from the leukocytes of most horses (Telford *et al.*, 1993) however it has been associated with immunosuppression in foals, respiratory tract disease and poor performance (Browning *et al.*, 1988). EHV-2 has also been suggested to have a role in reactivation of latent EHV-1 and EHV-4 (Welch *et al.*, 1992).

Compared to what is known about EBV and KSHV, little is known about EHV-2 infection in the natural host. EHV-2 can be grown in tissue culture however and the genome has been fully sequenced (Telford *et al.*, 1995). The genome of EHV-2 is 184 kb in size and consists of a 149kb unique region and a 17.5 kb direct repeat. The GC content of the genome is similar in the unique (58%), and repeat (55%) regions of the genome. In contrast, the HVS genome has a GC content of 35%

in the unique region and 71% in the terminal repeat region. The genome is collinear with HVS and EBV, but shares more similarity with HVS. Genes which have no counterparts in HVS are named E1 to E10. Eight proteins in the genome have counterparts in HVS but not in EBV. Four of these have sequence homology (ORFs 10, 11, 70, and 74) and four have positional homology (ORFs 12, 13, 28 and 74).

HVS and EBV ORF 51 genes both encode predicted glycoproteins and are positional homologues of gp350/220 of EBV. Only one protein of EHV-2 has a homologue in EBV but not in HVS. This is the BCRF1 homologue ORF E7, encoding an IL-10 like protein (Vieira *et al.*, 1991). The amino acid sequence is highly conserved between the human, EBV and EHV-2 proteins. EHV-2 encodes three potential G-protein coupled receptors. One of these encoded by ORF 74, is related to the mammalian chemokine receptor for IL-8 and to ORF 74 of HVS, suggesting a similar function. ORF E1 has homology with the human ß chemokine receptor CKR1 and with the HCMV US28 protein, which also binds ß chemokines (Neote *et al.*, 1993). EHV-2 encodes two proteins which are potentially involved in the prevention of apoptosis of infected cells. ORF E4 encodes a bcl-2 homologue similar to the BALF1 homologue of EBV. The EBV gene BALF1 has been shown to not have any apoptotic activity of its own, however it acts as a negative regulator of the *bcl-2* homologue BHRF1 (Bellows *et al.*, 2002). It is not known if the E4 gene of EHV-2 is functional. The E8 ORF encodes v-FLIP that has been shown to bind to the caspase-8 prodomain and block Fas and TNFR1 induced apoptosis (Bertin *et al.*, 1997).

An unusual feature of the EHV-2 genome is that approximately a third of the sequence does not appear to encode proteins. This includes most of the terminal repeat and adjacent sequences and regions containing the inverted repeat sequences IR1 and IR2. Sequence analysis of these regions has revealed no homology to known ORFs. It may be that the regions are transcribed into highly spliced mRNAs, which are translated into proteins lacking cellular or viral homologues. It has also been suggested that these regions may be transcribed into non-translated RNAs, similar to the HVS HSURs or EBV EBERs, however these small RNAs are insufficiently conserved to allow identification of counterparts in EHV-2 by sequence analysis.

Clearly transcriptional analysis of these regions would be required to resolve the question of their function.

1.4.2 Bovine Herpesvirus- 4 (BoHV- 4)

Bovine herpesvirus-4 was first isolated in Europe from cattle with respiratory and ocular disease (Bartha *et al.*, 1966). The virus has since been isolated from cattle with abortion, metritis, pneumonia, diarrhoea and mammary pustular dermatitis, however the role of BoHV-4 in the pathogenesis of these diseases is not clear and BoHV-4 is not associated with any lymphoproliferative diseases. Bovine herpesvirus-4, in common with EHV-2, was originally classified as a betaherpesvirus due to its biological properties. It has now been reclassified as a gammaherpesvirus as a result of sequence analysis of parts of the virus genome (Bublot *et al.*, 1992).

BoHV-4 establishes persistent infections in its natural host (Osorio & Reed, 1983) and in experimentally infected rabbits (Osorio *et al.*, 1982). It is thought that the site of persistence is a cell of the macrophage/monocyte lineage, however little else is known about persistence in the host. BoHV-4 has been demonstrated in the cell fraction of milk from infected cattle and is thought that virus may be transmitted to calves by this route (Donofrio *et al.*, 2000). In contrast to other gammaherpesviruses such as EBV, BoHV-4 replicates in tissue culture and cell lines such as Georgia bovine kidney cells and baby hamster kidney cells can be used for its growth.

The genome of BoHV-4 has been completely sequenced and established as that of a gammaherpesvirus (Zimmermann *et al.*, 2001). The genome is collinear with other gammaherpesviruses and consists of a long unique region flanked by terminal repeats. The unique region contains two regions of multiple direct repeats, the number and length of these differing between isolates (Bermudez-Cruz *et al.*, 1997). The unique genes are named Bo1 to Bo17. In general the genome of BoHV-4 differs from other gammaherpesvirus genomes in a reduced number of potential transforming genes or cellular homologues. No cytokine or cytokine receptor encoding genes are present as are found in HVS, HHV-8 and EHV-2 and no G protein-coupled receptor homologues are present.

BoHV-4 contains two genes encoding potentially antiapoptotic proteins, a bcl-2 homologue (ORF 16) and a death effector domain containing v-FLIP gene (ORF 71). The v-FLIP gene has been shown to inhibit Fas and TNF 1 induced apoptosis in common with other v-FLIPs such as ORF E8 of EHV-2 and the KSHV v-FLIP. Also of interest is the fact that the virus contains two copies of the putative viral formylglycineamide ribotide amino transferase (v-FGARAT) at ORF 3 and ORF 75 in common with HVS and AlHV-1. Bo17 encodes a viral ß-1,6-N-acetylglucosaminyltransferase (ß-1,6GnT), which is transcribed during viral replication and is functionally active (Vanderplasschen *et al.*, 2000). No other virus is known to encode this gene and it has been suggested to be necessary for replication of the virus in mononuclear cells, or may be involved in viral immune escape. The gene Bo10 encodes a glycoprotein and is predicted to be spliced. The gene is positionally homologous to gp350/220 of EBV and the ORF 51 gene of HVS and EHV-2. Little is known about latent gene expression of BoHV-4, however a bovine macrophage cell line supporting persistent BoHV-4 infection has recently been established in order to investigate latency *in vitro* (Donofrio & van Santen, 2001). Since the genome of BoHV-4 is less complex than other gammaherpesviruses, and the virus can be grown in tissue culture, it has been suggested that BoHV-4 may be of use as a gene therapy vector. The availability of the rabbit as a small animal model has resulted in interest in using the genome to insert other gammaherpesvirus genes in order to investigate their *in vivo* function.

1.4.3 Ovine Herpesvirus-2 and Alcelaphine Herpesvirus-1

These viruses cause the disease malignant catarrhal fever in ruminants worldwide and will be discussed in detail in a later section.

1.5 Identification of New Gammaherpesviruses

The genes involved in gammaherpesvirus replication are highly conserved. This fact has been used to develop a technique called consensus PCR (VanDevanter *et al.*, 1996). The method relies on using highly conserved amino acid motifs contained in the DNA polymerase genes of herpesviruses as the basis for PCR amplification using

degenerate primers. Consensus PCR has been used to partially sequence the polymerase genes of several existing herpesvirus isolates and to characterise new herpesviruses similar to KSHV in macaque species (Rose *et al.*, 1997).

Recently several new ruminant gammaherpesviruses have been identified using consensus PCR. Herpesviral DNA was amplified from the spleen and peripheral blood leukocytes (pbl) of white tailed deer in an outbreak of malignant catarrhal fever (Li *et al.*, 2000). PCR amplification using primers specific for OvHV-2 or AlHV-1 failed to amplify viral DNA, however viral DNA was amplified using degenerate primers for a conserved region of a herpesviral DNA polymerase gene. The new herpesvirus sequence exhibited 82% identity to OvHV-2 and 71% to AlHV-1. The technique was used to detect a new bovine gammaherpesvirus bovine lymphotropic herpesvirus-1 (BLHV-1) (Rovnak *et al.*, 1998), which is thought to have a possible role in the pathogenesis of enzootic bovine leucosis caused by bovine leukaemia virus (BLV). A new herpesvirus of goats was identified using consensus PCR and named caprine herpesvirus-1 (Li *et al.*, 2001b), (Chmielewicz *et al.*, 2001). The virus is thought to be found worldwide. It is endemic in goats and has recently found to be associated with the disease malignant catarrhal fever in sika deer (Crawford *et al.*, 2002).

1.5.1 Porcine Lymphotropic Herpesviruses

A search for new gammaherpesviruses of pigs was initiated due to concerns over the use of porcine organs for xenotransplantation and the possible risk of transmission of porcine pathogens to the transplant recipient. The use of consensus PCR using primers designed against the herpesvirus DNA polymerase gene identified two novel porcine herpesviruses, which were named porcine lymphotropic herpesvirus (PLHV) 1 and 2 (Ehlers *et al.*, 1999). The viruses were identified from peripheral blood mononuclear cells (PBMCs) and spleen of pigs. Initial analysis of the amino acid sequence of the DNA polymerase genes of the two herpesviruses showed PLHV-1 shared 68% identity with alcelaphine herpesvirus-1 and ovine herpesvirus-2, and 67% identity with BLHV-1. PLHV-1 and PLHV-2 differ from each other by 8% at the amino acid level. PLHV-1 and 2 have not been found to be associated with any disease under natural conditions, however a porcine herpesvirus associated with post-transplant lymphoproliferative disease in miniature swine following allogeneic

haematopoietic stem cell transplantation (Huang *et al.*, 2001) was found to be identical to PLHV-1. In order to investigate the molecular mechanisms by which PLHV-1 may cause PTLD, sequence of the genome was required. This was difficult however, since neither a lytic cell culture system or a permanent cell line harbouring PLHV-1 was available.

A 73kb section of the PLHV-1 genome PLHV-1 sequence was obtained by PCR-based genome walking from the splenic DNA of a domestic pig that was PCR positive for PLHV-1 but not for PLHV-2 (Goltz *et al.*, 2002). The section of the genome sequenced showed a genome arrangement typical of a gammaherpesvirus, however a number of non-conserved genes were found. A homologue of EBV BALF1 and EHV-2 E4 is present; this ORF has homology with bcl-2. The PLHV-1 ORF A5 is related to ORF A5 of AlHV-1 and BILF1 of EBV. This ORF is predicted to encode a viral GPCR similar to ORF 74 of KSHV. The PLHV ORF A6 shows low homology to the positional homologues of AlHV-1 ORF A6, EBV BZLF1 and KSHV ORF K8. ORF A7 was most closely related to ORF A7 of AlHV-1 and ORF BZLF2 of EBV, and ORFA8 was most closely related to ORF A8 of AlHV-1 and BLLF1 of EBV.

ORF 3 of PLHV-1 encodes a homologue of the cellular enzyme FGARAT. Homologues are found in the same position in AlHV-1, EHV-2, HVS and HVA. It is not known if this virus also contains a FGARAT homologue at ORF 75 like HVS AlHV-1 and BoHV-1 as the sequence of this region was not known. At the amino acid level, almost all PLHV-1 genes were closely related to AlHV-1. The sequence of PLHV-1 gB was contained in the new sequence. Phylogenetic analysis of gB sequences confirmed the findings from analysis of the DNA polymerase sequence, that PLHV1 was closest to AlHV-1, OvHV-2, BLHV and CprHV-2.

The genome of PLHV-1 was found to be transcriptionally active in the lymph nodes of pigs with PTLD but not in normal pigs. This fact has been very useful in terms of study of PTLD, as it has enabled transcriptional analysis of areas of the PLHV-1 genome. Transcription of the PLHV-1 genes E4/BALF1, ORF 3 (v-FGARAT), ORF 8 (gB), ORF A5 (v-GPCR), ORF 29 (terminase), ORF 45 (IE protein), ORF 50a & b (IE protein), A7 (glycoprotein) was found in PTLD pigs. Splicing was demonstrated in ORF 50, ORF 29 and ORF A6. ORF A6 was spliced

into 3 exons in the same way as has been shown for EBV ORF BZLF1. PLHV-1 is thought to infect B cells in pigs, although this has not been definitively proven. It is thought that expression of ORFs such as the v-GPCR in PTLD may be involved in the induction of cell proliferation in the same way as the KSHV v-GPCR.

It is hypothesised that PLHV-1 might be involved in the pathogenesis of PTLD in miniature swine and therefore study of the sites of latency and transmission are planned in order to further characterise natural infection among pigs. PLHV-1 infection in pigs may be a useful large animal model system for human PTLD. Since the genome of PLHV-1 is closely related to that of AlHV-1 and OvHV-2, sequence and transcriptional analysis provides valuable information for comparison of the function and pathological mechanisms of these viruses.

1.6 Malignant Catarrhal Fever

Malignant catarrhal fever (MCF) is a disease of cattle, deer and other ruminants, characterised by lymphoproliferation and tissue necrosis (Reid *et al.*, 1984). The disease is almost invariably fatal and is found worldwide.

MCF is mostly found in two forms, both of which involve an asymptomatic carrier host acting as a reservoir of disease. In sub-Saharan Africa, the blue wildebeest acts as a reservoir of infection (Plowright, 1960). This form of the disease is referred to as wildebeest associated MCF (WA-MCF). In the rest of the world, sheep are thought to be the main reservoir of infection, therefore this form is referred to as non-wildebeest associated MCF (NWA-MCF) or sheep associated MCF (SA-MCF). MCF affected animals are not infectious to other animals in natural conditions and are thought be dead end hosts.

1.6.1 Importance and Incidence of MCF

Outbreaks of Wildebeest associated MCF in Africa frequently involve many cattle. The disease also occurs in zoos and game farms worldwide where wildebeest (*Connochaetes taurinus*) are kept. An outbreak of MCF in a Los Angeles zoological park resulted in the deaths of four exotic ungulates over a 2 month period (Meteyer *et al.*, 1989). Two Chinese water deer *(Hydropotes inermis)*, a gerenuk (*Litocranius*

walleri) and a red flanked duiker (*Cephalophus rufilatus)* died. It was thought that the source of infection was a newly purchased wildebeest bull.

Sheep associated MCF occurs worldwide wherever domestic sheep are kept. The susceptibility of affected species is variable; European cattle are relatively resistant and incidence is mainly sporadic, although infection can occur in outbreaks over weeks, months or even years in which substantial losses may occur (Twomey *et al.*, 2002) (Otter *et al.*, 2002). Other species such as Bali cattle are particularly susceptible and must be kept separately from sheep if losses are to be avoided. (Pierson *et al.*, 1979, Taneichi *et al.*, 1986). Bison are also particularly susceptible to MCF and large outbreaks of SA-MCF have occurred in bison feedlots in North America (O'Toole *et al.*, 2002). The problem is such that MCF is the most important acutely fatal infectious disease in the commercial bison industry. Similar problems have occurred with SA-MCF in zoos where sheep have been kept with a range of susceptible ruminants (Li *et al.*, 1999b). MCF is also a problem in deer. When the farming of red deer was first established in Europe and New Zealand, major outbreaks occurred affecting up to 50% of the herd (Reid *et al.*, 1979), however it generally now occurs sporadically affecting individual animals. Père David's deer are particularly susceptible to MCF and attempts to farm this species of deer were abandoned due to losses occurring from MCF (Reid, 2000). MCF has been seen in numerous outbreaks in white-tailed deer (Brown & Bloss, 1992, Shulaw & Oglesbee, 1989), and substantial losses have occurred. MCF has also been reported affecting pigs, but only in Scandinavian countries (Loken *et al.*, 1998). MCF was first reported in Japan in 1956 (Fujimoto, 1958). Enzootics of SA-MCF in beef cattle in Japan killed 5 cattle, all of which had been housed adjacent to two ewes and their lambs (Taneichi *et al.*, 1986).

1.6.2 Clinical Signs of MCF

The incubation period of MCF in natural infection varies from 3 to 8 weeks and after artificial infection averages 22 days. The clinical course of MCF varies from acute to chronic (Reid *et al.*, 1984) and is almost invariably fatal, although there have been reports of recovered cases (O'Toole *et al.*, 1997, Penny, 1998). Malignant catarrhal fever is described as occurring in 2 forms; the "head and eye" form and the peracute

alimentary tract form, although these are extremes of a range of clinical signs, (Radostits *et al.*, 1994).

In disease in cattle the "head and eye form" predominates (Figure 1.6). The signs seen are anorexia, persistent pyrexia, lymphadenopathy, profuse mucopurulent nasal discharge, dyspnoea due to obstruction of the nasal cavities with exudate, ocular discharge and congestion of the scleral vessels, corneal opacity, necrosis of the nasal mucosa and large patches of necrosis on the muzzle. Later nervous signs such as incoordination can be seen and in longer duration forms of the disease exudative discharge from the skin can be seen. Occasionally horns and hooves can be shed. Typically the disease lasts 3 - 7 days and rarely to 14 days, however in cattle there have been reports of the disease taking a more chronic course. A chronic case of MCF was reported by O'Toole *et al.*, (1997) which survived for 280 days until euthanasia. Recovered cases remain persistently infected with OvHV-2 and are usually left with corneal oedema and a chronic keratitis. In deer the more acute form of the disease is often seen. The symptoms are those of acute gastrointestinal disease, haemorrhagic diarrhoea, depression and anorexia. The affected animal deteriorates rapidly over 12 – 24 hours and often animals will simply be found dead.

Unusual presentations of MCF can be seen. A case of MCF was reported in a yearling bull in which the main sign observed was an exudative discharge from the skin, along with pyrexia and anorexia (Holliman *et al.*, 1994). The bull died 17 days after the initial onset of symptoms and tested PCR positive for OvHV-2 DNA. Histological findings in the skin were also typical of MCF, making a false positive PCR result less likely.

1.6.3 Cause of MCF and Disease Transmission

WA-MCF is caused by the gammaherpesvirus Alcelaphine Herpesvirus 1 (AlHV-1) (Plowright, 1960), which has been propagated *in vitro* in monolayered cells of bovine origin (Plowright, 1963). All adult wildebeest tested have been shown to have indirect immunofluorescence (IIF) and virus neutralising (VN) antibodies to AlHV-1 (Plowright, 1967). Most wildebeest calves are infected with AlHV-1 before 3 months of age and virus is excreted in the nasal and lachrymal secretions (Mushi, 1980). Virus has also been isolated from wildebeest foetuses, suggesting infection *in utero*

Figure 1.6 The Clinical Signs of Malignant Catarrhal Fever

A

B

The photographs illustrate some of the clinical signs of cattle with the head and eye form of MCF. A shows catarrhal inflammation and erosion of the nasal mucosa, whilst B shows a purulent nasal discharge (Photographs kindly supplied by Dr Hugh Reid, Moredun Research Institute).

may be possible. It is thought that perinatal calves are the main source of infectious AlHV-1 where WA-MCF is found. In Africa the seasonal occurrence of MCF normally parallels the calving period of wildebeest. Wildebeest cows may also excrete virus in late pregnancy (Plowright, 1965) or if stressed by confinement, therefore these may also be a source of infection (Rweyemamu *et al.*, 1974).

Sheep were long suspected to be associated with the incidence of NWA-MCF, due to circumstantial evidence linking them with outbreaks of the disease (Piercy, 1954). Sheep sera were later shown to have IIF antibodies to AlHV-1. The pattern of immunofluorescence observed was similar to that detected from sera for wildebeest, or from AlHV-1 infected cattle (Rossiter, 1981). The sheep sera were, however not virus neutralising. It was also shown that cattle with non-wildebeest associated MCF possessed IIF antibodies to AlHV-1 which were not virus neutralising (Rossiter, 1983). Immunoblotting analysis of sera against the structural proteins of AlHV-1 showed that wildebeest sera reacted with all proteins, but sheep sera and sera from cattle with NWA-MCF only reacted with a subset of the proteins (Herring *et al.*, 1989). It was concluded from these studies that sheep carry a virus antigenically related to AlHV-1. It has not been possible to culture a virus from the tissues of animals with SA-MCF, however it has been possible to culture lymphoblastoid cell lines from the tissues of MCF affected animals (Reid *et al.*, 1983). These cell lines transmit disease and viral DNA with homology to AlHV-1 has been detected in the cell lines (Bridgen & Reid, 1991). As a result of this finding SA-MCF was proposed to be caused by a herpesvirus, which was named Ovine herpesvirus-2 (OvHV-2).

A PCR test was developed from the limited sequence available (Baxter *et al.*, 1993). This has been useful in the diagnosis of MCF and also for testing of sheep for the presence of OvHV-2 sequences. Use of the PCR test confirmed that most sheep are infected with the virus and it is thought that most sheep worldwide are infected with OvHV-2 (Baxter *et al.*, 1997). Transmission of SA-MCF is associated with lambing time (Selman *et al.*, 1978). It is assumed that transmission of OvHV-2 to susceptible animals from sheep occurs after close contact with sheep, however transmission has occurred when animals are separated from sheep by a considerable distance (Weaver, 1979).

Other ruminant gammaherpesviruses similar to AlHV-1 and OvHV-2 have been identified, some of which have been associated with disease. MCF has been recently found in sika deer infected with a recently identified gammaherpesvirus of goats named caprine herpesvirus-2 (Section 1.5). A new gammaherpesvirus causing MCF in white tailed deer has recently been identified (Li *et al.*, 2000); the virus was closely related to both AlHV-1 and OvHV-2. MCF killed 5 out of 6 white tailed deer in a North American zoo and the sixth deer was euthanased. The herpesvirus was identified in each deer by PCR, although the reservoir host for the virus was not identified. Many other wild ruminants have been found to be carriers of gammaherpesviruses and are resistant to clinical disease. A gammaherpesvirus has been isolated from a roan antelope and named hippotragine herpesvirus-1 (HipHV-1) (Reid & Bridgen, 1991). A virus Alcelaphine herpesvirus 2 has been isolated from hartebeest (Reid & Rowe, 1973) and topi (Mushi *et al.*, 1981). An outbreak of an MCF like disease in Barbary Red deer was recently reported to be associated with a herpesvirus closely related to AlHV-2, which was thought to have originated from Jackson's hartebeest (Klieforth *et al.*, 2002).

A recent study of ruminants in a wildlife park showed that antelope of the genus *Oryx* were infected with a gammaherpesvirus. Scimitar-horned oryx and gemsbok had antibodies which cross-reacted with AlHV-1 and a high prevalence of gammaherpesvirus DNA was detected using primers against herpesvirus DNA polymerase (Flach *et al.*, 2002). A herpesvirus was isolated from a scimitar-horned oryx which caused MCF when inoculated into rabbits. It is not known if this herpesvirus causes natural MCF.

1.6.4 Control of MCF

At present no vaccine is available for MCF, therefore control is based around separation of susceptible animals from wildebeest or sheep especially around lambing or calving time. This is particularly difficult in zoos and wildlife parks, where separation of susceptible animals from wildebeest or sheep may not be practical. It is also becoming more difficult in Africa to remove cattle from areas where wildebeest graze due to settlement and use of traditional grazing areas for arable land. A vaccine to protect susceptible animals against SA-MCF and WA-MCF

would therefore be useful, however so far attempts to develop an effective vaccine have failed. In the case of SA-MCF cattle and sheep are often grazed together, yet the incidence of disease is generally sporadic. Outbreaks can however, occur and in these cases advice is to dispose of the whole sheep flock for slaughter (Reid, 2000). Of particular concern is the new herpesvirus of white tailed deer (Li *et al.*, 2000) (Kleiboeker *et al.*, 2002), as a reservoir host for this virus has not been identified thus making planning of control measures difficult.

A number of different approaches to development of a vaccine have been used. Initial attempts at vaccination of cattle against WA-MCF involved inoculation of living or inactivated tissue culture grown AlHV-1, combined with Freund's incomplete adjuvant on two occasions at 6-8 weeks apart. High levels of virus neutralising antibody was detected in vaccinated animals, however no protection against disease was observed after challenge with virulent AlHV-1. This indicated that humoral immunity alone was not important in protection against AlHV-1 (Plowright *et al.*, 1975). In addition, the avirulent WC11 strain of AlHV-1 did not reliably protect cattle against virulent virus (Plowright, 1968). Rabbits were vaccinated with the inactivated virulent C500 strain of AlHV-1 (Russell, 1980) and 4 out of 6 rabbits remained healthy up to 5 months after two challenges with C500 AlHV-1. This raised hopes that vaccination with inactivated C500 AlHV-1 may be of use in protecting cattle. More recently a study by Mirangi (1991) showed that it was possible to protect cattle against the virulent C500 strain of AlHV-1, by a vaccination programme using a strain of AlHV-1 (707K) isolated from WA-MCF affected cattle in America. Protection was not always achieved however, and multiple injections of the 707K virus were required to induce protection which in itself risked induction of an MCF-like disease.

1.6.5 Experimental Transmission of MCF

WA-MCF was first transmitted to cattle from blood of a normal wildebeest, thus establishing wildebeest as a source of infectivity for cattle (Mettam, 1923). WA-MCF can also be transmitted experimentally to cattle and to rabbits by inoculation of intact cells or blood from infected animals (Plowright, 1968). This

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form of MCF has also been passed to hamsters, rats and guinea pigs (Jacoby *et al.*, 1988). Sheep associated MCF has also been passed to hamsters (Buxton *et al.*, 1988).

Experimental transmission of SA-MCF proved more difficult and transmission from affected cattle to healthy cattle is achieved only irregularly (Selman *et al.*, 1978). Experimental transmission of SA-MCF to rabbits was first achieved by intraperitoneal injection of a suspension of spleen and mesenteric lymph nodes from an MCF affected deer (Buxton & Reid, 1980). Transmission of SA-MCF from cattle and deer to cattle, deer, rabbits and hamsters was later reported (Reid *et al.*, 1986). MCF in rabbits is similar in clinical course to the disease seen in cattle and deer, therefore rabbits have proved very useful as an experimental model of disease. Transmission of SA-MCF has been maintained continuously in rabbit to rabbit passages with more than 100 serial transfers and infectivity demonstrated in culture cells, but it has not been possible to identify the causal agent in these cells (Reid *et al.*, 1984). MCF has also been recently transmitted experimentally from sheep to Japanese deer by housing in close contact with sheep (Imai *et al.*, 2001).

1.6.6 Pathology of MCF and Post Mortem Findings

Malignant catarrhal fever is a multisystemic disease involving many tissues. Post mortem findings vary between cases. In animals which die of acute MCF gross post mortem changes may not be obvious, however generally the gross changes involve extensive inflammation and necrosis of the mucosal surfaces (Jubb *et al.*, 1993). The nasal turbinates, larynx and trachea are hyperaemic and often have extensive catarrhal exudates. There are often erosions and ulcerations of the mucosa of the respiratory tract and the tongue may be ulcerated. The lungs are usually oedematous and emphysaematous, but in peracute cases they may appear normal. Lymph nodes are generally enlarged and oedematous. The liver and spleen are congested and enlarged. Haemorrhage, hyperaemia and thickening of the bladder wall are seen. In a study of Indonesian swamp buffalos with MCF, epicardial and pericardial haemorrhages were consistently found, however this finding is not often reported in domestic cattle (Hoffmann *et al.*, 1984). Small foci of interstitial nephritis are sometimes seen in the kidneys. Erosions and ulcerations are also found in the

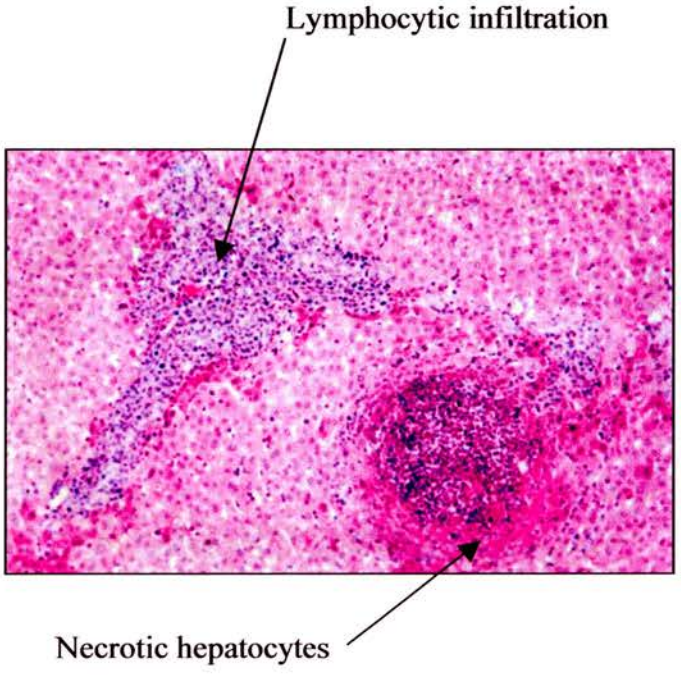
intestinal mucosa. The intestinal lesions tend to be more severe and extensive in deer, where they can extend from the duodenum to the rectum (Reid *et al.*, 1984).

Histologically, lesions are characterised by proliferation of lymphoid tissues, mononuclear cell infiltration of non-lymphoid tissues and inflammation including vasculitis and necrosis (Figure 1.7). A detailed study of the vascular lesions of MCF in calves was carried out (Liggitt & DeMartini, 1980a). Vascular lesions are found in the small and medium arteries of many tissues. Commonly affected were vessels in the gastrointestinal (GI) tract, oral epithelium, eye, kidney, liver, lung and brain. The principle lesion of the vasculitis was lymphoid cell infiltration of the adventitia, with medial and intimal changes. The infiltrating mononuclear cells were mostly lymphocytes and lymphoblasts and fewer monocytes and macrophages.

In epithelial tissues there were foci of lymphoid accumulation, epithelial cell degeneration and necrosis (Liggitt & DeMartini, 1980b). The lesions were found in the epithelia of the GI tract, biliary epithelium, conjunctiva, lachrymal, pancreatic and salivary gland ducts, respiratory tract, urinary bladder and choroid plexus. The infiltrating cells were mononuclear cells, mainly lymphocytes, lymphoblasts and macrophages. In cattle that survive MCF for a prolonged period or appear to recover, the vasculitis may progress to a proliferative arteritis, with proliferation of the smooth muscle cells of the vessel wall (O'Toole *et al.*, 1995). Severe ocular lesions such as erosive keratoconjunctivitis, lymphocytic retinitis and anterior uveitis are also typical of MCF (Whiteley *et al.*, 1985).

Study of the pathology of rabbits infected with SA-MCF (Buxton *et al.*, 1984), described interstitial accumulations of lymphocytes occurring in the early stages of disease, the proliferative response being hyperplastic rather than neoplastic with retention of normal tissue architecture. Tissue destruction occurred only in the later stages of disease. In general rabbits infected with SA-MCF have more severe lesions in the mesenteric lymph nodes compared with rabbits infected with AlHV-1, which have more severe lesions in the popliteal and submandibular lymph nodes. The spleen was enlarged in all cases of MCF in rabbits. Treatment of the rabbits with the T cell suppressor cyclosporin A did not affect the course of disease, showing the lymphoproliferative component of MCF may not be directly involved in pathogenesis.

Figure 1.7 Pathology of MCF



The photograph shows a haematoxylin and eosin stained section of the liver of a cow with MCF. The section shows lymphocytic inflitration and necrosis of hepatocytes.

1.6.7 Target Cells and Sites of Viral Replication

The tissues of rabbits infected with WA-MCF were tested for infectivity using calf thyroid cell monolayers (Patel & Edington, 1980, Rurangirwa & Mushi, 1982). Most infectivity was demonstrated in lymphocytes and it was therefore concluded that lymphocytes are the major target cell for AlHV-1 in rabbits. A further study of WA-MCF in rabbits found that the spleen was positive for virus antigen by indirect immunofluorescence (IIF) at 2-3 days post infection. Virus was also recovered from spleen, lymph nodes and thymus at 8 and 12 days post infection. This was taken to suggest a primary cycle of virus replication in the spleen (Edington & Patel, 1981).

Tissue sections and cultured lymphocytes from rabbits infected with WA-MCF were examined for presence of virus by *in situ* hybridisation (Bridgen *et al.*, 1992). Small numbers of virus infected cells were consistently detected in the submandibular lymph nodes, whilst other tissues showed no evidence of viral DNA. The virus from MCF infected rabbit tissues was titred and higher titres of virus were found in lymph node, spleen and lung of infected animals, whilst only low titres were found in kidney and peripheral blood lymphocytes (pbls). The number of lymphocytes positive for AlHV-1 DNA by *in situ* hybridisation increased after 24 hours in culture, suggesting viral replication may be suppressed by the host.

1.6.8 Phenotypical Analysis of the Lymphoproliferative Lesions of MCF

The phenotype of the infiltrating cells in the vascular lesions of MCF has been studied using monoclonal antibodies specific for bovine lymphocyte markers. In a study by Ellis *et al.*, (1992) frozen sections of choroid plexus, choroid rete and choroid of the eye were examined from a case of SA-MCF in a 1-year-old heifer, which was also infected with bovine viral diarrhoea virus (BVDV). The main infiltrating cell was found to be CD8+ cytotoxic T lymphocytes. In a separate study by Nakajima *et al.*, (1992), frozen sections of the kidney, bladder, lymph nodes, heart, brain and liver of a 2 year old cow with SA-MCF were examined and the cells phenotyped. The study found that in agreement with Ellis *et al.*, (1992), CD8+ lymphocytes were found infiltrating the blood vessels of the brain, heart and liver. The lymphoproliferation in the lymph nodes was also found to consist of the same

cell type, however the function of the CD8+ lymphocytes in the pathogenesis of the disease is not clear and it is not known if these cells were infected with virus.

1.6.9 Culture of Cell Lines From MCF Affected Animals

Lymphoblastoid cell lines can be derived in culture from the tissue of cattle and rabbits with both forms of MCF (Cook & Splitter, 1988, Reid *et al.*, 1989, Reid *et al.*, 1983). The first reported cell line was cultured from the mesenteric lymph node of a rabbit infected with SA-MCF (Reid *et al.*, 1983). The cell lines required feeder monolayers of foetal ovine kidney cells for their propagation and were cytotoxic to both primary cell cultures and cell lines as shown by chromium release assays. The cells had the morphology of large granular lymphocytes (LGL), which are often cytotoxic. Although no viral particles could be identified in the cell line, as few as 100 cells transmitted disease to other rabbits.

Subsequently lymphoblastoid cell lines were propagated from the tissues of cattle and red deer with SA-MCF (Reid *et al.*, 1989). These cell lines were derived from thymus, lymph node, spleen suspensions and also from cerebro-spinal fluid cells and cultured cornea. A cell line was also derived from a Père David's deer affected by MCF. Sometimes the cell lines survived only for a short time, however some cell lines could be maintained indefinitely as lymphoblastoid cell lines by providing feeder cell monolayers and IL-2 and some cell lines became independent of these factors. The cells had the morphology of large granular lymphocytes and exhibited cytotoxicity characteristic of natural killer cells, which are morphologically LGL (reviewed by Herberman & Ortaldo, 1981). In contrast to the cell lines derived from rabbits, one cell line from a red deer transmitted disease however none of the cell lines from cattle did. This has led to suggestions that in terminal stages of MCF the viral genome may be only partially conserved (Reid *et al.*, 1986).

Numerous speculations have been made as to the mechanisms involved in the pathology of MCF. Since infiltrating mononuclear cells have been identified in the lesions, the mechanisms have been suggested to be cell mediated immune responses to virally infected vascular epithelium (Liggitt & DeMartini, 1980a), (Taneichi *et al.*, 1986) or an autoimmune mechanism of graft versus host reaction. It was suggested by Plowright, (1968) that hypersensitivity to viral or viral antigens may be involved

in pathogenesis. Given the propagation of LGL cell lines from MCF affected animals, it has been proposed that the LGL may be a target cell for the disease (Reid *et al.*, 1984) and the pathology may result from dysregulation of the function of this cell type. This could cause the non-specific polyclonal T cell proliferation by loss of suppressor T cell activity of the LGLs, whilst the tissue necrosis might be caused by extension of the natural killer cell activity of the LGLs to normal tissues.

1.6.10 Further Characterisation of OvHV-2 Infected Cell Lines

The phenotype of a number of OvHV-2 infected cell lines is variable. OvHV-2 infected cell lines cultured from the tissues of cattle and deer with MCF were examined by Burrells & Reid, (1991). Cell lines from three out of four cattle were identified as CD4- CD8+, whilst the other cell line was CD4+ CD8-. Two cell lines were derived from deer and were CD4-CD8-. Bovine Cell lines of mixed CD4+ and CD8+ phenotype were identified by Schock *et al.*, (1998). All cell lines examined expressed a CD2 T cell marker. The pathology of MCF has been described as similar to the pathology induced by the viruses HVS and HVA (Hunt & Billups, 1979; Patel & Edington, 1980) and it has been suggested that similar pathological mechanisms may be involved. This view is reinforced by the fact that OvHV-2 infected LCLs have been observed to be similar to HVS and HVA infected T cell lines. In common with OvHV-2 infected cell lines, HVS and HVA infected cell lines expressed a T cell receptor, NK antigens and exhibited NK function (Kiyotaki *et al.*, 1988; Kiyotaki *et al.*, 1986). In addition, some HVS transformed cells do not shed infectious virus even after stimulation (Meinl *et al.*, 1997). HVS infected cell lines from rhesus monkeys were CD4+ CD8+ (Meinl *et al.*, 1997). HVS transformed human T cells had an activated T cell phenotype and both CD4+ and CD8+ cell lines were identified (Mittrucker *et al.*, 1992).

Infection of human T cell clones with HVS was reported to modify the cytokine secretion profile (De Carli *et al.*, 1993). All infected cell lines were induced to express IFNγ and IL-2, and some cell lines were induced to express TNF-ß and IL-4. The cytokine transcription of OvHV-2 infected T cell lines was studied for comparison (Schock *et al.*, 1998). All cells transcribed IFNγ, TNFα, IL-4 and IL-10. No mRNA for IL-2 was detected. It was observed that the secretion profile shares

similarities with HVS infected cell lines and with anergic T cells (reviewed by Schwartz, 1997). As a result of these observations, it was proposed that OvHV-2 infected T cell lines are activated anergic T cells and that the maintenance of anergy of peripheral T cells is altered by the virus during MCF (Schock *et al.*, 1998). The secretion of IL-10 was observed to be of interest as both EBV and EHV-2 encode IL-10 homologues and it was proposed that the IL-10 might be of viral origin.

Since both HVS and EBV express viral proteins which interfere with cell signal transduction molecules, particularly the non-receptor protein tyrosine kinases, these mechanisms were investigated in relation to the behaviour of AlHV-1 and OvHV-2 infected lymphocytes. It was shown that the src kinases Lck and Fyn were constitutively active in OvHV-2 and AlHV-1 infected LCLs and not in non-infected lymphocytes (Swa *et al.*, 2001). Lck and Fyn are src kinases involved in the initiation of T cell activation via the T cell receptor (TCR) or other structures such as CD2, CD4, CD8 and the IL-2 receptor (Qian & Weiss, 1997; van Leeuwen & Samelson, 1999). They are the first known signalling molecules to be recruited to the TCR after antigen or other stimulation of the TCR. The kinases become activated themselves by phosphorylation of their activation motifs and go on to phosphorylate immunoreceptor tyrosine-base activation motifs (ITAMs) on TCR CD3 and ξ chains. This then leads to a series of signalling events leading to T cell gene transcription and expression of an activated T cell phenotype. It is thought that viral proteins are responsible for the changes in Lck and Fyn in infected cells, however an amino acid homology search for putative binding factors for host signalling molecules failed to reveal any candidates in the AlHV-1 genome.

1.6.11 Sequence Analysis of AlHV-1

Since the pathology and lymphoproliferative syndromes found in rabbits or marmosets infected with HVS are similar to those found in MCF, the AlHV-1 genome was completely sequenced in order to identify and compare the pathogenesis associated genes (Ensser *et al.*, 1997) (Figure 1.8).

The strain of AlHV-1 sequenced was the virulent C500 strain. The virus was confirmed to be a gammaherpesvirus of the genus rhadinoviridae. The genome is

essentially collinear with other rhadinoviruses such as HVS, KSHV and MHV-68, although differences were found in the regions between the blocks of conserved genes. There were no homologues of known cytokine related or transformation associated genes of KSHV and HVS. Since the AlHV-1 genome was fully sequenced, sequencing of a large part of the porcine gammaherpesvirus PLHV-1 has also been achieved (Goltz *et al.*, 2002) (Section 1.5.1). This herpesvirus is most similar to AlHV-1 and OvHV-2 and it has been suggested that in future a new genus may contain all three of these gammaherpesviruses, along with BLHV-1 and the new gammaherpesvirus of goats, CprHV-2.

The genes of AlHV-1 were numbered according to the counterpart in HVS, whilst the open reading frames (ORFs) with no homology to HVS genes were named A1 to A10. The conserved genes are arranged in four blocks collinear to HVS, whilst the non-conserved ORFs are found in between these blocks. The putative viral transactivators encoded by ORF 50 (BLRF1 of EBV) and ORF 57 (BMLF1 of EBV) are only weakly conserved, however they have been shown to be functional transactivators (Frame, 2001; Devi, 2001). ORF 73 shares structural similarity to ORF 73 of HVS and KSHV. A fusion protein derived from this cDNA is recognised by antisera from AlHV-1 infected animals (Lahijani *et al.*, 1995). Recently antibodies to OvHV-2 ORF 73 have been identified in sheep by screening an expression library from OvHV-2 infected cattle LCLs with sheep sera (Coulter & Reid, 2002). The ORF 75 gene is homologous to formylglycineamide ribotide amino transferase (FGARAT). This gene and its homologue ORF 3 at the left hand end of the genome are conserved in other gammaherpesviruses as described in section 1.3.1. AlHV-1 does not have a homologue or a positional homologue of ORF28. The only other gammaherpesvirus sequenced so far that shares this property is PLHV-1. An ORF 49 homologue is also absent, however this is present in PLHV-1.

The ORFs A1 to A4 are located at the left-hand end of the genome between the terminal repeats and the conserved gene ORF 3, which is at the left end of the first gene block. This position is equivalent to the transformation-associated proteins of HVS, STP and Tip (Duboise *et al.*, 1998), however none of the genes A1 to A4 show any similarity to them. The A1 open reading frame is predicted to encode 97aa

Figure 1.8 The Genome Organisation of Alcelaphine Herpesvirus 1

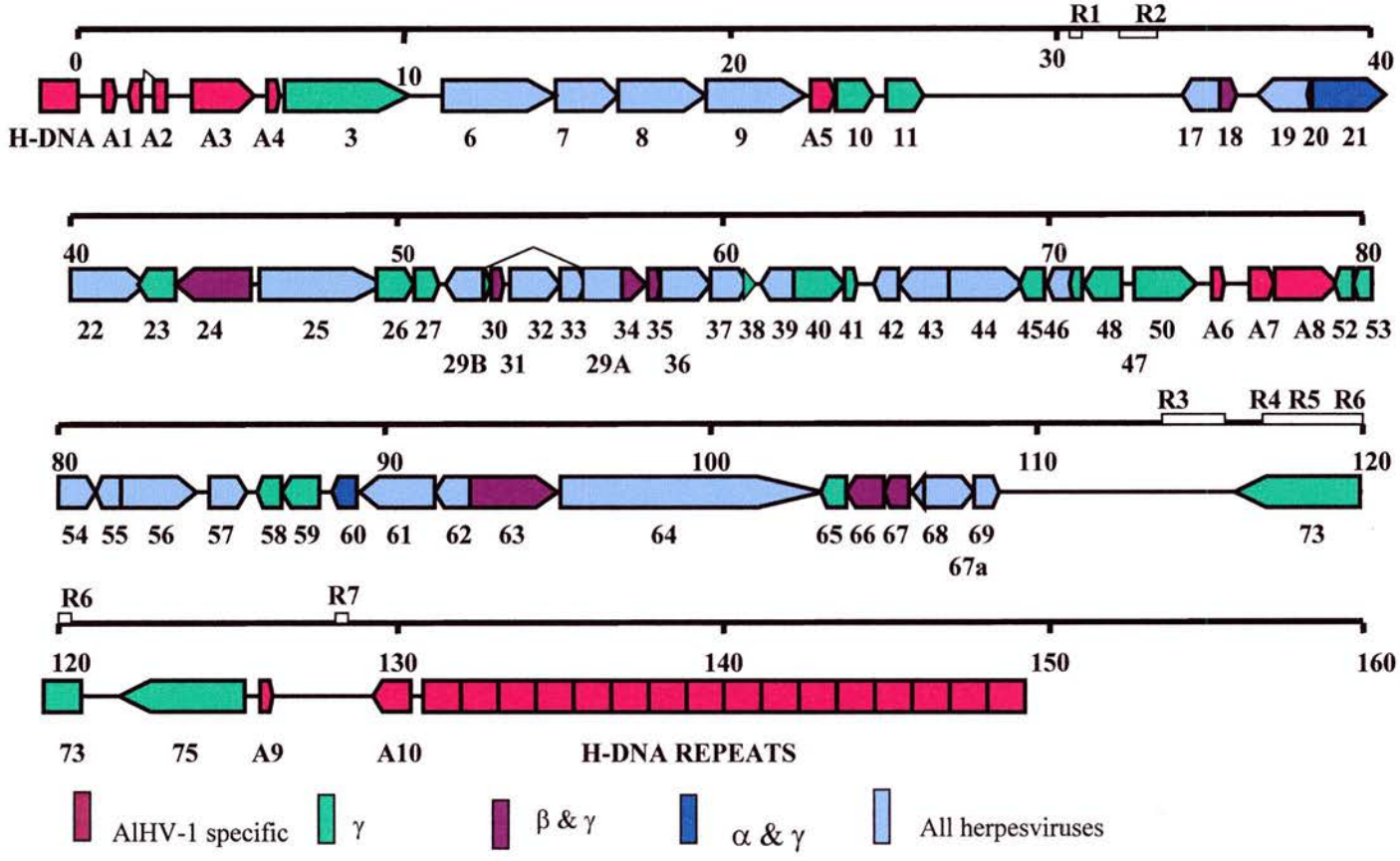


Figure 1.8 The Genome Organisation of Alcelaphine Herpesvirus 1. The diagram shows the linearised genome of AlHV-1. The open reading frames are represented as block arrows, which are coloured according to the conservation of the open reading frame among the three subfamilies (α, ß and γ) of herpesviruses. Major repetitive regions of the genome are marked R1 to R7. The genome length is shown in Kbp (Adapted from Ensser *et al.*, (1997)).

protein. A1 has a TATA box-like sequence upstream and polyadenylation site downstream, providing further evidence that A1 is a real ORF. The A2 protein is encoded by a spliced transcript. It has a motif similar to nuclear localisation signals and the basic domain of the stress-induced transcription factor ATF3 (Chen *et al.*, 1996). ORF A3 is homologous to the semaphorin gene family (Ensser & Fleckenstein, 1995). Semophorins were originally described as a family of related transmembrane and secreted proteins, acting as repulsive signals for growth cone guidance in embryonic development (Kolodkin *et al.*, 1993). Truncated genes of similar structure are found in poxviruses (Comeau *et al.*, 1998) but not in any other herpesviruses. It has been proposed that poxvirus semophorins play a role in the immune system as natural immunosuppressants that are secreted from virus infected cells, therefore would have a role in reducing the host immune response to infection.

ORF A4 encodes a protein of 121 aa with a putative signal peptide cleavage site, indicating the protein may be secreted. ORF A5 is a homologue of the EBV gene BILF1 and EHV-2 E6. This gene is however, missing in HVS and KSHV. This might suggest that the gene may have been present in an ancestral gammaherpesvirus, but has been lost in primate rhadinoviruses. It may encode for a protein with the characteristics of a G protein coupled receptor, as sequence analysis shows seven putative transmembrane domains. The A5 gene may therefore be involved in the induction of cell proliferation in the same way as the v-GPCR of KSHV (Arvanitakis *et al.*, 1997).

The region of the genome including ORFs A6, A7 and A8 is similar in its arrangement of genes to EBV. A6 may therefore encode a regulatory protein like the BZLF-1 gene of EBV (Packham *et al.*, 1990). ORF A7 shows a weak amino acid similarity to ORF 51 of HVS and EHV-2, including a potential signal peptide cleavage site between the arginine and leucine residues at positions 19 and 20. ORF A8 is predicted to encode a glycoprotein of 683 aa. It is positionally equivalent to gp350/220 of EBV and therefore may be a candidate for interaction with a host cell receptor for AlHV-1. ORF A9 encodes a protein of 168 aa with homology to the bcl-2 family. Despite being located at a different genomic position to bcl-2 homologues of HVS, EBV and KSHV, A9 is similar in size to the other viral bcl-2 homologues. It contains a well conserved BH1 region and a conserved hydrophobic

carboxy terminus. AlHV-1 ORF A4.5 encodes an additional bcl-2 homologue which was not identified in the original sequence. This ORF is homologous to the BALF1 ORF of EBV, which encodes a second bcl-2 homologue in EBV. The BALF1 protein has been found to negatively regulate the antiapoptotic activity of BHRF1 (Bellows *et al.*, 2002), therefore it is possible that the A4.5 protein of AlHV-1 may negatively regulate the activity of A9 in a similar way. Orf A10 has the potential to encode a 472 aa glycoprotein.

Despite similarities of MCF to HVS induced pathology and lymphoproliferative syndromes, AlHV1 contains no homologues of the HVS transforming proteins STP and Tip. This suggests that though the phenotype of ALHV-1 infected cell is similar to those infected with HVS, different viral effector molecules act to achieve this similar phenotype. The complete sequence of AlHV-1 is a considerable aid to the study of AlHV-1 pathogenesis, as it will enable the functions of possible pathogenesis associated genes to be examined, as well as the production of recombinant viruses and antibodies, to study the expression of viral proteins.

1.6.12 Identification of AlHV-1 Virulence Genes *In Vivo*

When AlHV-1 is passaged through bovine cell cultures the C500 isolate loses its ability to cause MCF in the laboratory model, the rabbit. This attenuation is associated with an alteration in the genome (Handley *et al.*, 1995) affecting ORF 50, A7 and A3. It has been shown that ORF 50 is not expressed in the attenuated form of AlHV-1 (Wright *et al.*, 1997), and ORF A6 and A7/8 are deleted. ORF 50 has homology to EBV transcriptional transactivator (Rta) and HVS ORF 50 transcriptional transactivator. It has been demonstrated that AHV ORF 50 is a functional transactivator acting on the ALHV-1 immediate early (ORF 57) and delayed early (ORF 6) promoters (Frame, 2001) and may be an important control element in AlHV-1 gene expression. As described, ORF A3 encodes a semaphorin homologue (Ensser & Fleckenstein, 1995), which may be important in immune evasion. ORF A7 and A8 are in an equivalent position in the genome to BLLF1 of EBV, which encodes a glycoprotein gp350 mediating attachment of EBV to the B cell receptor CD21. A8 therefore may be a candidate for interaction with a host cell

receptor for AlHV1. It is not known which ORFs are responsible for the attenuated *in vivo* phenotype of passaged AlHV-1, however each ORF involved in the rearrangement has potential importance in disease pathogenesis.

1.6.13 Molecular Studies of OvHV-2

The lack of a tissue culture system for OvHV-2 has made working with the virus difficult and attempts to identify a virus in cultured cell lines have failed. The first OvHV-2 sequence to be identified was a DNA clone derived by hybridisation of AlHV-1 probes to a genomic OvHV-2 phage λ library (Bridgen & Reid, 1991). OvHV-2 virus has been difficult to detect by hybridisation analysis, however development of a PCR test (Baxter *et al.*, 1993) has now enabled sites of OvHV2 infection in sheep to be studied and is also used for diagnosis of clinical cases of MCF. The PCR test was designed from sequence from a clone with homology to AlHV-1 ORF 75 and to EBV ORF 75 or BNRF1. The primers used amplify a 238bp fragment of OvHV-2 viral DNA. Other methods of diagnosis of OvHV-2 infection have been developed, such as a competitive-inhibition ELISA (Li *et al.*, 1994). However PCR was found to be the most sensitive test available and has made diagnosis of cases in the incubation period possible (Muller-Doblies *et al.*, 1998). The use of ELISA was not successful in diagnosing acute cases of SA-MCF, possibly due to the animal having insufficient time to mount an immune response (Li *et al.*, 1995).

Studies regarding the acquisition of OvHV-2 infection by lambs are conflicting. In a study by Baxter *et al.*, (1997) OvHV-2 DNA was detected in nasal secretions and epithelial tissues from all lambs tested during the first 2 months following birth. However Li *et al.*, (1995), found that only a small percentage of the pbls of lambs were OvHV-2 positive by ELISA and by PCR up to 12 weeks old. At 3.5 months old however, the percentage of lambs PCR positive rose sharply to 87%. Antibody and viral DNA were not found in samples from pre-suckling lambs, suggesting that transplacental transmission of viral is a rare event if it does occur. Suckling lambs were found to acquire high levels of antibody by passive transfer in colostrum. Virus DNA was detected in colostrums and milk by PCR testing and it is thought that virus may be transmitted by this route. It was found that removing lambs

from the flock at 2.5 months old prevented OvHV-2 infection, therefore it was suggested that the positive PCR signals sometimes seen before this point resulted from passive transfer of maternal leukocytes which are then eliminated.

The production of OvHV-2 free sheep by separating lambs from the ewes by two months old has enabled the transmission of OvHV-2 between adult sheep to be studied (Li *et al.*, 1999a). Here it was found that adult sheep that have been maintained OvHV-2 free are just as susceptible to OvHV-2 infection as are lambs. Development of quantitative methods of PCR such as competitive PCR and fluorogenic PCR have enabled quantitation of viral DNA in samples from asymptomatic sheep and from animals with SA-MCF (Hua *et al.*, 1999; Hussy *et al.*, 2001). In one 7 month old OvHV-2 infected sheep, higher levels of virus DNA were found in the nasal secretions than in pbls. This suggested enhanced viral replication in the nasal, pharyngeal or possibly ocular epithelium. A study of OvHV-2 in the nasal secretions of sheep found that levels of shedding of OvHV-2 DNA were undetectable until 5 months old. Shedding peaked at 7 months old and then remained relatively stable. This was taken to suggest that the main source of transmission of OvHV-2 to susceptible species was adult sheep rather than neonatal lambs (Li *et al.*, 2001a). In one study adult sheep pbl were separated into different cell types and analysed for the presence of OvHV-2 DNA (Baxter *et al.*, 1997). The OvHV-2 DNA was detected in the B lymphocyte fraction of the cells and OvHV-2 DNA was not detected in the T lymphocyte or macrophage fractions. In contrast, the culture of OvHV-2 infected cell lines of the LGL phenotype from cattle, deer and rabbits with MCF has led to suggestions that LGLs are the target cell for OvHV-2 in these species (Reid *et al.*, 1983) (Reid *et al.*, 1989). Evidently more *in vivo* studies are required to identify the sites of replication and latency of OvHV-2 in its natural host and in MCF affected animals.

1.7 Project Outline

Little is known about Ovine herpesvirus-2 infection due to the lack of a replicative tissue culture system for growing virus. The resources available for the study of ovine herpesvirus-2 are; the rabbit as a model system and the lymphoblastoid cell lines cultured from virus infected animals. Since at least rabbit cell lines transmit

disease, it may be that whole virus is present in this cell line, however little is known about the mechanisms of virus persistence in these cell lines. The availability of sequence of OvHV-2 would be a huge step in the study of this virus, as this would enable the production of antibodies and probes with which to study expression of viral genes in the *in vivo* rabbit model. This would enable the identification of viral virulence genes and the testing of their function *in vivo* using recombinant viruses.

The aims of this project were:

1. To characterise the state of the viral genome in OvHV-2 infected cell lines.
2. To generate OvHV-2 viral sequence by construction of a cosmid library using DNA extracted from OvHV-2 infected cell lines, which could be probed with walking probes in order to generate a series of overlapping cosmids spanning the viral genome.
3. To study in detail an area of viral sequence of interest resulting from the cosmid sequence. This is likely to come from the left hand end of the virus, where the genes involved in virulence were expected to be located, by analogy with other gammaherpesviruses.

Chapter Two: Materials & Methods

2.1 Molecular Cloning

2.2 Southern Analysis of DNA

2.3 Gardella Gel Analysis

2.4 Polymerase Chain Reaction

2.5 Construction of a Cosmid Library

2.6 Sequence Analysis

2.7 RNA Analysis

2.8 Protein Analysis

2.9 Antibody Production

2.10 Analysis of Eukaryotic Cells

2.1 Molecular Cloning

Molecular cloning methods were based on the protocols described in Sambrook *et al.*, (1989). All common buffers and stock solutions are described in appendix 1, located at the end of materials & methods.

2.1.1 Digestion of DNA with Restriction Enzymes

Restriction enzymes were obtained from either Invitrogen or New England Biolabs and were used with the supplied buffers according to the manufacturer's instructions. Restriction enzyme digests were typically carried out for 2 hours at 37°C in a 20-200μl volume, using 10 units enzyme per 1-10μg of DNA.

2.1.2 Purification of DNA

DNA was purified after enzyme reactions by extraction with phenol : chloroform. A mixture of phenol (equilibrated to pH 7.8 with TE), chloroform and iso-amyl alcohol in the ratio 25:24:1 was added to an equal volume of DNA solution and mixed by vortexing. After centrifuging for 5 minutes at 10,000 x *g*, the aqueous phase was removed into a clean tube and the process repeated until no protein debris remained at the interphase between the aqueous and organic phases (usually just once). The sample was then extracted once with chloroform in order to remove any remaining phenol and the sample concentrated by ethanol precipitation.

2.1.3 Ethanol Precipitation of Nucleic Acids

DNA (or RNA) was precipitated by addition of 0.3 volumes 7.5M ammonium acetate or 0.1 volume 3M sodium acetate (pH 5.2), and 2.5-3 volumes of ice cold 96% (v/v) ethanol (VWR). The solution was mixed and incubated at –20°C for 1 hour. The precipitated DNA was then recovered by centrifugation at 12,000 x *g* for 5 minutes. The DNA was washed in 70% ethanol, air dried for 10 minutes and resuspended in the appropriate volume of TE or dH_2O.

2.1.4 Agarose Gel Electrophoresis

DNA or RNA were analysed by gel electrophoresis on 0.5-1% agarose gels containing 0.5μg/ml (w/v) ethidium bromide (Sigma), in TAE or TBE buffer.

Samples were made up to a 10-20 μl volume with TE, and mixed with 1/10 volume loading buffer (15% (w/v) Ficoll type 400, 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanol) or 0.25% (w/v) Orange G (Sigma) in dH_2O). Electrophoresis was carried out at 70V in a horizontal tank (Bio-Rad) containing TAE or TBE buffer. The size of DNA bands was estimated by comparison with molecular weight standards (1Kb plus DNA ladder or 0.24 - 9.5Kb RNA ladder, Invitrogen). Bands were visualised under ultraviolet (UV) light using a short wave UV transilluminator (UVP).

2.1.5 Purification of DNA from Agarose Gels by Electro-Elution

DNA bands were excised from agarose gels using a sterile scalpel blade under UV light. The gel slice was then inserted into clean sterile dialysis tubing (Visking size 2-18/32", Medicell) and 300μl TAE added. Any air bubbles were removed and the tubing sealed at both ends. The dialysis tubing was then immersed in TAE in a mini gel tank and electrophoresis carried out for 1 hour at 70V. The current was then reversed for 30s to remove any DNA attached to the tubing, and the TAE solution containing the DNA removed into a clean microfuge tube. The DNA was then phenol : chloroform extracted and concentrated by ethanol precipitation.

2.1.6 Purification of DNA from Agarose Gels Using a QIAquick Gel Extraction Kit (QIAGEN)

This was done according to the manufacturer's instructions. The gel slice containing DNA was weighed, and 3 volumes buffer QG added to 1 volume of gel (100mg~100μl). The sample was then incubated for 10 minutes at 50°C, vortexing every 2 minutes, until the gel slice had completely dissolved. One gel volume of isopropanol was then added and the sample added to the QIAquick column, before centrifuging for 1 minute at 10,000 x *g* to bind the DNA to the membrane. The DNA was then washed once with buffer QG (0.5ml) and once with buffer PE, before eluting the DNA by adding 50μl of buffer EB, incubating for 1 minute and centrifuging for 1 minute at 10,000 x *g*.

2.1.7 Quantification of DNA

DNA concentration was assessed using a fluorometer (DyNAQUANT 200, Hoefer, Amersham Biosciences). DNA was diluted 1 : 1000 in 0.2M HCl, 10mM Tris (pH7.4), 1mM EDTA containing 0.1μg/ml bisbenzimide (Hoechst H33258, Amersham Pharmacia). Binding of the Hoechst dye to DNA results in a shift in the emission spectrum of the dye that can be measured using a photodetector. The DNA concentration is calculated by comparing the signal to that obtained with a 100ng/ml DNA standard.

2.1.8 De-Phosphorylation of Linearised DNA

In order to prevent re-circularisation during ligation reactions, the 5' phosphate group on each end of linearised vector DNA was removed using calf intestinal alkaline phosphatase (CIAP, Roche). Following restriction enzyme digest, the DNA was incubated with 20U CIAP for 20 minutes at 37°C and the DNA purified by either phenol : chloroform extraction and ethanol precipitation, or using the CONCERT rapid PCR purification system (Invitrogen).

2.1.9 Ligation of DNA

Ligations were set up with using approximately 50ng linearised plasmid DNA (cloning vectors used are shown in appendix 5) and variable amounts of fragment DNA depending on the size, such that the ratio of plasmid : insert was 1 : 4. The reaction contained 1 x T4 DNA ligase buffer (50mM Tris-HCl, 10mM $MgCl_2$, 10mM DTT, 1mM ATP, 25μg/ml BSA, New England Biolabs), and 6 Weiss units T4 DNA ligase in a reaction volume of 20μl. All ligations included negative controls whereby the fragment was omitted from the reaction in order to check for self-ligation of the plasmid. Ligation was performed overnight at 14°C.

2.1.10 Transformation of Competent Bacteria with Plasmid DNA

Eschericia coli bacteria (Epicurian coli XL-1 Blue competent cells, Stratagene) were transformed according to the manufacturer's protocol. Competent cells were thawed on ice and a 50μl aliquot transferred to a pre chilled 15ml falcon polypropylene tube. ß-mercaptoethanol was added to a concentration of 25mM and the tubes incubated

on ice for 10 minutes with regular agitation. A 1μl aliquot of the ligation reaction was then added and the cells mixed before incubation on ice for 30 minutes. The cells were then heat pulsed at 42°C for 45 seconds, and the tubes incubated on ice for a further 2 minutes, before 0.9ml of pre-warmed SOC medium was added. The tubes were then incubated at 37°C for 1 hour with shaking. A 100μl sample of the mixture was then spread on LB agar plates containing antibiotic (100μg/ml ampicillin or 50μg/ml kanamycin, Sigma). The remaining mixture was centrifuged for 2 minutes at 4,000 x *g*, and the pellet resuspended in 150μl medium before spreading on an agar plate. The plates were incubated overnight at 37°C, after which the plates were stored at 4°C for up to 4 weeks.

2.1.11 Small Scale Preparation of Plasmid DNA (Mini-Preps)

Mini-preps were usually performed using the alkaline lysis method. A bacterial colony was picked from the plate using a flamed loop and grown up in 10ml Luria-Bertani (LB) medium, with the appropriate antibiotic, overnight at 37°C in an orbital shaker. A sample of the culture was then streaked onto an LB-agar plate with the appropriate antibiotic and this was stored at 4°C after growing overnight at 37°C. The bacteria were then centrifuged to a pellet at 4,000 x *g* for 10 minutes. The pellet was resuspended in 200µl of solution I (50mM glucose, 25mM Tris.HCl pH 8.0, 100µg/ml RNase; (Invitrogen) and 1 mg/ml lysozyme; (Sigma)), and transferred to a microfuge tube. A 400µl volume of solution II (0.2N NaOH, 1% (w/v) sodium dodecyl sulphate; (SDS)) was then added and the solution mixed by inverting, before incubating for 5 minutes on ice. A 300µl volume of solution III (5M potassium acetate, 12% (v/v) glacial acetic acid pH 4.8) was then added and the solution mixed by inversion before incubating for 5 minutes on ice.

The solution was then centrifuged for 5 minutes at 10,000 x *g*, and the resulting supernatant transferred to a new microfuge tube before adding 500µl of isopropanol alcohol (IPA, VWR). The tube was then incubated for 15 minutes on ice and then centrifuged for 5 minutes at 10,000 x *g*. The resulting pellet was dried and resuspended in 200µl TE. A 100µl volume of 7.5M potassium acetate was then added and the solution incubated for 15 minutes on ice before centrifuging at 10,000 x *g* for 5 minutes. The supernatant was then transferred to a new tube and

600µl 95% ethanol (VWR) added and the solution mixed before incubating for 1 hour at -20°C. The solution was then centrifuged for 5 minutes at 10,000 x *g* and the pellet of plasmid DNA washed in 70% ethanol, before centrifuging at 10,000 x *g* for 10 minutes. The supernatant was then removed and the pellet air dried before resuspending in 20µl TE pH 8.0. The samples were stored at 4.0°C and analysed by restriction enzyme digestion.

2.1.12 Preparation of DNA for Sequencing

Preparation of plasmid DNA for sequencing and small scale preparation of cosmid DNA was done using a QIAprep spin mini prep kit (QIAGEN) according to the manufacturer's instructions. A single colony was used to inoculate 10ml LB medium containing the appropriate antibiotic and grown overnight at 37°C with shaking. The bacteria were then pelleted at 1,500 x *g* for 5 minutes, before resuspension in 250µl buffer P1. Buffer P2 (250µl) was then added and the sample mixed by inversion and incubated for 5 minutes at room temperature. Buffer P3 (350µl) was then added and the sample mixed before centrifuging for 10 minutes at 10,000 x *g*. The resulting supernatant was decanted into a QIAprep spin column and the DNA bound to the membrane by centrifugation at 10,000 x *g* for 1 minute. The membrane was then washed in 500µl buffer PB, and 750µl buffer PE before elution of the DNA in 50µl Tris pH 8.5. For elution of cosmid DNA, the elution buffer was heated to 70°C before use.

2.1.13 Concentration of Cosmid DNA For Sequencing

The concentration of cosmid DNA purified using the QIAGEN miniprep kit was usually low. In preparation for sequencing, cosmid DNA was concentrated using YM-100 centrifugal filter devices (Millipore) according to the manufacturer's instructions. The cosmid DNA was made up to a volume of 150-200µl with diH_2O and loaded into the filter device, before centrifuging for 12 mins at 500 x *g*. The filter device was then inverted and the concentrate eluted by centrifugation for 3 mins at 1,000 x *g*.

2.1.14 Large Scale Preparations of DNA (Maxi-Prep)

Large scale preparations of plasmid DNA were performed using anion exchange columns (Endofree Plasmid Maxi Kit, QIAGEN) according to the manufacturer's instructions. An overnight bacterial culture (250ml) was centrifuged at 6,000 x *g* for 15 minutes at 4°C and the pellet resuspended in 10ml of buffer P1 (50mM Tris pH8.0, 10mM EDTA, 100μg/ml RNase A). A 10ml volume of buffer P2 (200mM NaOH, 1% (w/v) SDS) was then added and the solution mixed by gentle inversion, before incubation for 5 minutes at room temperature. A 10ml volume of ice-cold buffer P3 (3M potassium acetate pH5.5) was then added and the sample mixed gently by inversion before incubation for 10 minutes on ice. The mixture was then filtered using a QIAfilter cartridge, after which 2.5ml endotoxin removal buffer (buffer ER) was added and the sample mixed before incubation for 30 minutes on ice. A QIAGEN-tip 500 anion exchange column was equilibrated using 10ml buffer QBT (750mM NaCl, 50mM MOPS pH7.0, 15% isopropanol, 0.15% Triton X-100), and the filtered lysate added to the equilibrated column. The column was then washed twice with 30ml buffer QC (1M NaCl, 50mM MOPS pH7.0, 15% isopropanol) and the DNA eluted in 15ml buffer QN (1.6M NaCl, 50mM MOPS pH7.0, 15% isopropanol). The DNA was precipitated by adding 0.7 volumes isopropanol and pelleted by centrifugation at 15,000 x *g* for 30 minutes at 4°C. The pellet was washed with 70% ethanol before air drying and resuspending in 200μl TE.

2.1.15 Preparation of Plasmid DNA by Caesium Chloride Density Gradients

A bacterial colony was grown in 10ml LB broth supplemented with the appropriate antibiotic for 8 hours then inoculated into 400ml LB with antibiotic and grown overnight. The bacteria were then pelleted by centrifugation at 6,000 x *g* for 5 minutes, before resuspension in 20ml glucose solution (50mM glucose, 25mM Tris.Cl, 10mM EDTA) with 5mg/ml lysozyme (Sigma). The suspension was incubated for 10 minutes at room temperature, then 40ml mini preps solution II (Section 2.1.11) added. The mixture was incubated for 5 minutes on ice, before the addition of 20 ml ice cold mini preps solution III (Section 2.1.11). After mixing by inversion, the sample was then centrifuged for 5 minutes at 6,000 x *g* and the

resulting supernatant passed through 4 sheets of gauze to remove any particulate matter. The solution was precipitated by the addition of 0.6 volumes of isopropanol followed by incubation on ice for 30 minutes and the precipitate then pelleted by centrifugation at 6,000 x *g* for 5 minutes.

The pellet was air dried and resuspended in 6ml TE pH 8, after which 3ml 7.5M ammonium acetate was then added and the solution incubated on ice for 30 minutes. The sample was centrifuged for 10 minutes at 8,000 x *g* and the resulting supernatant transferred to a new tube. The sample was then precipitated by the addition of 18ml ice cold 95% (v/v) ethanol and incubation at –20°C for one hour. The precipitated DNA was pelleted by centrifugation at 8,000 x *g* for 10 minutes. The DNA was resuspended in 500μl TE pH 8 and transferred to an Ultraclear disposable ultracentrifuge tube (Beckman). A 200μl volume of 10mg/ml ethidium bromide solution was added and the tube filled to the top with a 100% (w/v) solution of caesium chloride in TE. The tube was weighed and a balance tube made to the same weight. The two tubes were then heat-sealed. The tubes were then loaded into the Vti70 rotor and centrifuged overnight at 260,000 x *g*. After centrifugation the tubes were carefully removed from the rotor and fixed over a UV light box. The thick band of plasmid DNA was then removed using an 18G needle and syringe.

2.1.16 Removal of Ethidium Bromide and Caesium Chloride from The DNA Preparation

An equal volume of isoamyl alcohol was added to the DNA solution and mixed by vortexing. The sample was then left to stand until it separated into a pink organic upper phase and a colourless aqueous phase. The organic phase was removed and the procedure repeated until no pink colour was visible in the organic phase (4 – 6 times), indicating removal of the ethidium bromide. The caesium chloride was then diluted by the addition of 3 volumes of TE pH 8 and 8 volumes of 95% (v/v) ethanol. The solution was stored overnight at 4°C and the solution precipitated by centrifugation at 10,000 x *g* for 15 minutes at 4°C. The precipitated DNA was then washed in 70% ethanol before resuspending in TE pH 8.

2.1.17 Storing Bacterial Cultures as Glycerol Stocks

Glycerol stocks were made by growing bacterial cultures to log phase and adding glycerol to 20% (v/v), before mixing and storing at -80°C.

2.1.18 Large Scale Preparation of Cosmid DNA

Large-scale purification of cosmid DNA was carried out using a QIAGEN large construct kit according to the manufacturer's instructions. This protocol was similar to the maxi prep kit but involved an exonuclease digestion step whereby contaminating genomic DNA and damaged cosmid DNA were removed from supercoiled cosmid DNA. Buffers were the same as those used in the EndoFree plasmid maxi kit unless stated. A 500ml overnight bacterial culture was pelleted by centrifugation at 6,000 x *g* for 15 minutes at 4°C and the bacterial pellet resuspended in 20ml buffer P1. Buffer P2 (20ml) was added and the sample mixed gently before incubation at room temperature for 5 minutes. Buffer P3 (20ml) was then added and the sample mixed before incubation on ice for 10 minutes. The sample was then centrifuged at 20,000 x *g* for 30 minutes at 4°C and the supernatant filtered to remove any particulate matter. The DNA was then precipitated by addition of 0.6 volumes isopropanol and pelleted by centrifugation at 15,000 x *g* for 30 minutes at 4°C. The DNA pellet was washed with 70% (v/v) ethanol, air dried and redissolved in 9.5ml buffer EX (50mMTris pH8.5, 10mM $MgCl_2$). ATP-dependant exonuclease (200μl) and 100mM ATP (300μl) were then added and the solution incubated at 37°C for 1 hour. A 10ml volume of buffer QS (1.5M NaCl, 100mM MOPS pH7.0, 15% isopropanol) was added to the solution and the solution added to an equilibrated QIAGEN-tip 500 ion exchange column. The column was washed twice with buffer QC and the DNA eluted with 15ml buffer QF (1.25M NaCl, 50mM Tris pH8.5, 15% isopropanol) at 65°C. The DNA was precipitated with 0.7 volumes of isopropanol before pelleting by centrifugation at 15,000 x *g* for 30 minutes at 4°C. The DNA pellet was washed in 70% ethanol before drying in air and resuspending in 200μl TE.

2.2 Southern Analysis of DNA (Southern Blotting)

2.2.1 Extraction of High Molecular Weight DNA from OvHV-2 Infected Cells

OvHV-2 infected cells were centrifuged for 5 minutes at 4,000 x *g* and washed twice in PBS before resuspension at $5x10^7$ cells/ml in TE. Extraction buffer (0.5% (w/v) SDS, 0.1M EDTA, 0.2M Tris.HCl pH8.0) (10 volumes) was then added and RNase (Sigma) added to a final concentration of 20µg/ml before incubation at 37°C for 1 hour. Proteinase K (Roche Molecular Biochemicals) was added to a final concentration of 100µg/ml and the solution incubated overnight at 56°C. After incubation overnight, the DNA solution was transferred to a polypropylene tube and extracted twice with phenol : chloroform and once with chloroform as described (Section 2.1.2). Since high molecular weight DNA is easily sheared, modifications to the phenol : chloroform extraction protocol were made. The DNA solution was mixed with the phenol : chloroform gently on a rotating wheel instead of vortexing and wide bore pipette tips were used. The solution was precipitated by adding 0.3 volumes 7.5M ammonium acetate and 3 volumes 95% ethanol. The resulting precipitate of DNA was spooled using a Pasteur pipette and washed twice with 70% (v/v) ethanol before drying in air and resuspending in TE pH 8.0 at 1ml per 5 x 10^6 cell equivalents. If necessary, the sample was incubated at 37°C to aid dissolving the DNA into solution. The DNA was then quantified using the fluorometer as described and a sample analysed by electrophoresis on a 0.7% (w/v) TAE agarose gel.

2.2.2 Electrophoresis and Southern Transfer of DNA

A 1% agarose gel was loaded with 5-10µg of DNA /lane and electrophoresis carried out at 80V until the dye front had migrated 8-10 cm down the gel. The gel was then photographed under UV light and rinsed in distilled water. The DNA was denatured by soaking the gel twice in 0.5M NaOH, 1M NaCl for 20 minutes on a rotary platform, before neutralising in 0.5M Tris.HCl pH 7.5, 1.5M NaCl for 2 washes of 20 minutes. The DNA was then transferred overnight to a nylon membrane (Magnagraph Nylon Transfer Membrane, Osmonics) by capillary transfer using 10 x

SSC. After transfer, the DNA was UV cross-linked to the membrane using the Stratalinker (Stratagene) on the "auto crosslink" setting and either stored at –20°C or pre-hybridised.

2.2.3 Pre-Hybridisation

Before pre-hybridising the 1kb DNA ladder was cut off the membrane and stained, by soaking in 1M acetic acid for 10 minutes, then in staining solution (0.2% methylene blue, 0.4M acetic acid, 0.4M sodium acetate). Excess stain was removed by washing in distilled water and the DNA ladder air dried. The membrane was pre-hybridised for at least 2 hours at 65°C, in 20ml 6 x SSC, 0.05 x BLOTTO (5% non-fat dried milk) or in 100ml of a solution containing 5 x Denhardt's solution, 4 x SSC, 200μg/ml denatured sheared salmon sperm DNA (Sigma), 0.1% SDS) in a hybridisation oven (Techne Hybridiser HB-1).

2.2.4 Radiolabelling of DNA Probes

DNA probes for hybridisation were labelled using a Random Primed DNA labelling kit (Roche) according to the manufacturer's instructions. The DNA probe (25ng) was denatured by heating to 100°C for 10 minutes, cooled on ice and added to a solution containing 25μM dATP, dCTP, dTTP, dGTP, 5μg hexanucleotide primer, 50μCi α^{32}PdCTP (Amersham Biosciences) and 2U Klenow DNA polymerase in a 20μl total volume. The reaction was incubated at 37°C for 40 minutes. Unincorporated nucleotides were then removed from the probe using a Nick sephadex G-50 column (Amersham Biosciences) and the labelled DNA eluted in 400μl TE pH7.5. The radiolabelled probe was denatured in 0.1 volumes 3N NaOH for 5 minutes at room temperature, then neutralised in 0.05 volumes 1M Tris pH 7.5 and 0.1 volumes 3N HCl. Then probe was then added to enough pre-hybridisation buffer to cover the membrane (10-20ml) and the membrane hybridised overnight at 65°C.

2.2.5 Membrane Washes

After hybridisation in order to minimise non-specifically bound probe, the membrane was washed in 5 x SSC, 0.5% SDS for 2 x 15 minutes at room temperature, then in 1 x SSC, 0.5% SDS for 2 x 15 minutes at 37°C, finally in 0.1 x SSC, 1% SDS for 3 x

15 minutes at 65°C. The membrane was then covered in Saran wrap and exposed to x-ray film (Kodak X-Omat AR, Sigma) at -70°C.

2.2.6 Use of Ultrahyb Hybridisation Buffer

In some situations, Ultrahyb sensitive hybridisation buffer (Ambion) containing 50% formamide was used for pre-hybridisation and hybridisation. A 10-12 ml volume was used depending on the size of the membrane. Pre-hybridisation and hybridisation carried was carried out using the same buffer at 42°C. Due to the increased sensitivity of this buffer, 1/10 of the radiolabelled probe was used. Membranes hybridised in Ultrahyb were washed at 42°C twice for 5 minutes in 2 x SSC, 0.1% SDS and twice for 15 minutes in 0.1 x SSC, 0.1% SDS before exposing to X-ray film as before.

2.2.7 Stripping of Radioactive Probe from Nylon Membranes

To remove radioactive probe from nylon membranes, boiled distilled water was poured on the membrane and the membrane soaked for 5 minutes. This procedure was usually repeated three times.

2.3 Gardella Gel Analysis

2.3.1 Preparation of Gardella Gels

Gardella gels were run as described by (Gardella *et al.*, 1984). The gel consisted of non-denaturing gel portion composed of 0.75% (w/v) agarose in TBE, forming the main body of the gel. A space was left for the denaturing part of the gel, which contained the wells. This gel contained 0.75% (w/v) agarose in TBE, 2% SDS, and 1mg/ml proteinase K (Roche) and was poured just before loading the gel.

2.3.2 Gardella Gel Loading and Electrophoresis

The cells used were fresh OvHV-2 infected LCLs obtained from Dr Hugh Reid (Moredun Research Institute) (Section 2.10.1). Positive controls used were fresh S11 cells (a B cell line infected containing MHV-68 in both latent and lytic forms) (Usherwood *et al.*, 1996) or fresh BCP-1 cells (KSHV infected B cells containing

both the lytic and latent forms of KSHV) (Boshoff *et al.*, 1998). Cells for analysis (2 x 10^6 of each type) were washed once in PBS before resuspending in 50µl loading buffer (15% Ficoll, 2 units RNase /ml (Sigma), 0.01% bromophenol blue in TBE). A small sample of 1µg Cosmid A8 (Supercos1-MW containing the left-hand end of MHV-68) was also loaded onto the gel as a control. After loading the cells were then overlain with denaturing gel and electrophoresis was then carried out at 4°C, at 0.3V/cm (20V) for 3 hours, then at 7.5V/cm (110V) for 17 hours.

After electrophoresis the gel was rinsed in TBE and stained by soaking in a solution of 0.5µg/ml ethidium bromide in TBE for 30 minutes at room temperature. Unbound ethidium bromide was then removed by soaking the gel in distilled water for 20 minutes. The stained gel was then visualised under UV light and photographed. The position of the DNA on the gel was then identified by Southern blotting as described (Section 2.2).

2.4 Polymerase Chain Reaction (PCR)

2.4.1 Components of PCR Reactions

PCR reactions were typically made up to a 100µl total volume containing 1 x PCR reaction buffer (20mM Tris pH8.4, 50mM KCl, Invitrogen), 1.5mM $MgCl_2$, 250µM of each of dATP, dGTP, dCTP, dTTP (Ultrapure dNTP set, Amersham Biosciences) 0.2pmol of each primer, 100-500ng DNA template and 1U *Taq* DNA polymerase (Invitrogen). Samples were made up to the correct volume using RNase free water (Sigma). PCR primers were obtained from MWG-Biotech and primers used are shown in appendices 2 and 3. Negative control reactions contained distilled water instead of template. PCR reactions were carried out in thin walled 0.5ml tubes (ThermoHybaid) in an Omnigene thermal cycler (ThermoHybaid). A modified "hot start" cycle was used to increase specificity of products. Reactions were heated to 94°C for 5 minutes, then held at the annealing temperature until the *Taq* enzyme was added. The PCR program then consisted of 30-40 cycles of 1 min annealing at temperature specific to primer, extension at 72°C for 1 min/Kb of product and 30s denaturing at 94°C. The reactions were then incubated at 72°C for a further 8 minutes before cooling to room temperature.

DNA for cloning or sequencing was amplified using *PfuTurbo* DNA polymerase (Stratagene). PCR reactions contained *PfuTurbo* DNA polymerase reaction buffer (20mM Tris pH8.8, 10mM KCl, 10mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.1mg/ml BSA), 0.2pmol of each primer, 100-500ng DNA template, 250μM each of dATP, dGTP, dCTP and dTTP (Ultrapure dNTP set, Amersham Biosciences) in a final volume of 100μl. Reaction conditions were similar to those used for PCR amplification with *Taq* DNA polymerase.

2.4.2 PCR Amplification of Long or Difficult Templates

PCR of long or difficult templates was performed using Expand Long Template PCR System (Roche) according to the manufacturer's instructions. This involves use of an enzyme mix containing thermostable *Taq* DNA polymerase and a proof reading polymerase. Reactions contained PCR buffer 3 (1 x PCR buffer with 2.25mM $MgCl_2$ and detergents), supplemented with 1.5μl 50mM $MgCl_2$ (Invitrogen) and final concentrations of 500μM dTTP, dGTP, dATP, and dCTP (Ultrapure dNTP set, Amersham Biosciences), 0.2pmol of each primer, 100-500ng template and 2.6U DNA polymerase mix in a 50μl total volume. The reactions were made up using two master mixes, one containing dNTP's, primer and template, the other containing DNA polymerase mix, buffer, and $MgCl_2$. The two components were mixed immediately before cycling and the reactions overlain with mineral oil. The enzyme was used to amplify cDNA where the product was expected to be over 1kb in length using a cycle of 2 mins at 94°C, followed by 40 cycles of denaturation at 94°C for 10s, annealing at the temperature determined by the primers for 30s, elongation 68°C for 45s/kb product and finally elongation at 68°C for a further 7 minutes.

PCR amplification of long templates was also performed using *TaqPlus Long* DNA polymerase (Stratagene). This was a mixture of a proof reading DNA polymerase (*Pfu* DNA polymerase, Stratagene) and *Taq* polymerase (*Taq* 2000, Stratagene). The concentrations of nucleotides, primer and template used were the same as for *Taq* DNA polymerase. Reactions also contained either 1 x *TaqPlus Long* low salt buffer (20mM Tris-HCl pH8.75, 10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 1% Triton X-100, 0.1mg/ml BSA) or 1 x *TaqPlus Long* high salt buffer (20mM Tris-HCl pH9.2, 60mM KCl, 2mM $MgCl_2$). Low salt buffer was used to

amplify templates up to 5kb and high salt buffer was used to amplify templates over 5kb in length. Cycling conditions were similar to those used for amplification using *Taq* DNA polymerase, except an extension time of 30s to 1min/kb was used.

2.4.3 Use of Expand Long Template PCR System to Amplify Genomic DNA

The enzyme was used to amplify DNA extracted from OvHV-2 infected cells using a cycle of denaturation for 2 minutes at 92°C, followed by 10 cycles of denaturation at 92°C for 10s, annealing at 62-65°C for 30s and elongation for 20-25mins at 68°C. This was followed by 10-15 cycles of denaturation at 92°C for 10s, annealing at 62-65°C for 30s and elongation at 68°C for 20-25 mins plus an additional elongation time of 20s for each cycle.

2.4.4 Genome Walking by Splinkerette PCR

Splinkerette PCR is a method of extending from known to unknown sequence by amplification of DNA sequences which lie between a single known primer and a nearby restriction site (Devon *et al.*, 1995).

2.4.5 Generation of Linker Molecules for Splinkerette PCR

Linker molecules were generated as described in (Devon *et al.*, 1995). Oligonucleotides used are shown in appendix 4. The splinktop and enzyme specific bottom oligonucleotides were resuspended in Tris pH 7.4 and 5mM $MgCl_2$ at 300μM, then 10μl of each oligonucleotide (one splinktop oligo and one splink bottom oligo) was mixed and heated to 90°C, before cooling at room temperature for 10-20 minutes.

2.4.6 Generation of Ligated Splinkerette Molecules

DNA extracted from OvHV-2 infected cell lines (2μg) was digested with the restriction enzyme required, in a 100μl reaction. The digested DNA was purified using a CONCERT Rapid PCR purification kit (Invitrogen) as described, resulting in a 50μl end volume. The digested DNA (4μl) was then ligated to annealed splinker

molecules (6μl) in a 20μl reaction at 15°C overnight. The ligated molecules were then used as a template for PCR reactions.

2.4.7 Splinkerette PCR Program

The program used involved combined hotstart and touchdown components to increase specificity of the reaction. The program comprised one cycle of heating at 45°C for 8 minutes, denaturation at 95°C for 5 minutes followed by cooling to the first annealing temperature of 71°C. The temperature was held at this point until *Taq* polymerase enzyme was added. This was followed by elongation at 72°C for 2 minutes, denaturation at 94°C for 1 minute and annealing at 69°C for 1 minute. This cycle was repeated 5 times, decreasing the annealing temperature by 2 degrees each cycle until the annealing temperature was 61°C. The cycle was then repeated 5 times with the annealing temperature at 61°C, 10 times with the elongation time increased to 4 minutes and 20 times with the extension time increased to 6 minutes. This was followed by elongation at 72°C for 8 minutes. This PCR program was then repeated using internal primers and the primary product as template. Any PCR products generated were cloned and sequenced as described (Section 2.4.9).

2.4.8 Purification of PCR Products

PCR products were purified using the CONCERT Rapid PCR Purification System (Invitrogen). The reaction was mixed with 400μl binding solution H1 before loading onto a spin cartridge. The DNA was bound to the membrane by centrifugation at 12,000 x *g* for 1 minute and the cartridge washed with wash buffer H2. A 50μl volume of TE heated to 65°C was then added, followed by incubation of the cartridge at room temperature for 1 minute. The DNA was then eluted by centrifugation at 12,000 x *g* for 2 minutes.

2.4.9 Cloning PCR Products

PCR amplification products produced using primers with restriction enzyme sites at the 5' end were digested with the appropriate restriction enzymes and then cloned into sequencing vectors such as pBluescribe KS II (Stratagene) (Appendix 5). PCR products without restriction sites were cloned using TA cloning vectors.

2.4.10 Cloning PCR Products using a pCR 2.1 TOPO TA Cloning Kit

PCR products were cloned using a pCR 2.1-TOPO TA cloning kit (Invitrogen) (Appendix 5) according to the manufacturer's instructions. This kit contains a linearised plasmid vector with single 3' – thymidine (T) overhangs and Topoisomerase I covalently bound. The TOPO cloning reaction contained a final concentration of 200mM NaCl, 10mM $MgCl_2$, 2μl fresh purified PCR product and 10ng plasmid. The reaction was mixed gently and incubated for 5-15 minutes at room temperature, before transforming One Shot TOP 10 chemically competent cells.

2.4.11 Transformation of One Shot TOP 10 Chemically Competent *Escherichia coli*

One Shot TOP 10 chemically competent cells (Invitrogen) were transformed according to the manufacturer's instructions. A 50μl aliquot of the cells was thawed on ice and 2μl of the TOPO cloning reaction was added. The sample was gently mixed and incubated on ice for 5 minutes, before heat shocking for 30s at 42°C. The tubes were then transferred to ice and 250μl SOC medium added before incubation at 37°C with shaking for 1 hour. Aliquots of 20μl, 50μl and the remainder of the sample were then spread onto LB-Amp plates to which 20μl 200mM isopropyl ß-D thiogalactoside (IPTG) and 40μl 2% (w/v) 5-bromo-4-chloro-3-indoyl-ß-D-galactoside (X-Gal) were added for blue/white selection of positive colonies. The plates were then incubated overnight at 37°C. Pure white colonies were picked and analysed by "mini prepping" and restriction enzyme digestion of the plasmid DNA.

2.4.12 TA Cloning using pGEM-T Easy Vector

PCR products were cloned using pGEM-T Easy vector (Promega) (Appendix 5) according to the manufacturer's instructions. A ligation mix was prepared containing 2 x Rapid Ligation Buffer, 50ng pGEM-T Easy Vector, 3μl PCR product and 6 Weiss units T4 DNA ligase, in a total volume of 10μl. The mixture was incubated at room temperature for 1 hour and a 2μl aliquot used to transform the XL-1 Blue strain of *E. coli*. After transformation the bacteria were plated out onto

LB-Amp plates to which IPTG and X-Gal had been added as described (Section 2.4.11) for blue/white selection of positive colonies.

2.5 Construction of a Cosmid Library

All restriction enzymes and buffers were from Invitrogen or New England Biolabs unless stated otherwise.

2.5.1 Cosmid Vector

Supercos1 (Stratagene) DNA, modified as described by (Cunningham & Davison, 1993) was provided by Dr A. Davison (University of Glasgow). SuperCos 1 contains genes conferring resistance to ampicillin and neomycin and two adjacent cos sites separated by an *Xba*I site. The region for inserting foreign DNA contains a single *Bam*HI site flanked by bacteriophage T7 and T3 transcriptional promoters and *Not*I and *EcoRI* sites in the configuration *EcoRI-Not*I-T7 promoter-*Bam*HI-T3 promoter-*Not*I-*EcoRI*. Supercos1 was modified by replacing the *Not*I fragment with a single synthetic oligonucleotide to give SuperCos 1MW, containing a cloning region with the configuration *EcoRI-Not*I-*Asc*I-*Pac*I-*Bam*HI-*Asc*I-*Pac*I-*Not*I-*EcoRI*.

2.5.2 Preparation of Cosmid DNA for Cloning

Cosmid DNA at 1μg/μl was digested in a number of steps as directed in the SuperCos 1 kit instructions provided by Stratagene. SuperCos 1MW DNA (25μg) was linearised by digestion with *Xba*I and digested cosmid DNA dephosphorylated using calf intestinal alkaline phosphatase (CIAP) (Roche) in 1 x dephosphorylation buffer in a 100μl reaction for 30 minutes at 37°C. The enzyme was then inactivated by adjusting the reaction to 15mM EDTA and heating at 68°C for 10 minutes. The sample was then extracted once with phenol : chloroform and once with chloroform before ethanol precipitating and resuspending in 25μl of TE pH8.0. The *Xba*I and CIAP-treated DNA was then digested with *Bam*H1 to give 2 cosmid arms of size 1.1 and 6.5kb. The DNA was then extracted once with phenol : chloroform and once with chloroform before ethanol precipitating and resuspending in 25μl distilled water (1μg/μl).

2.5.3 Partial Digestion of High Molecular Weight DNA for Cosmid Cloning

High molecular weight DNA extracted from OvHV-2 infected cells was partially digested with *Mbo*I such that the products were on average 40kb in size. This was done by preparing dilutions of *Mbo*I of 1/125, 1/250, 1/500, 1/1000 and 1/2000 in reaction buffer, 1µl of each dilution was used to digest 10µg of high molecular weight DNA in a 200µl reaction for 1 hour at 37°C. Samples were checked for digestion by electrophoresis of an aliquot alongside a 40kb size marker (Linearised SuperCos 1MW containing 40kb of the left-hand end of MHV-68), kindly provided by Dr A. MacRae. Samples which digested to produce DNA of the required size of approximately 40kb were extracted once with phenol : chloroform and once with chloroform, taking care not to vortex the samples, before dephosphorylating with calf intestinal alkaline phosphatase (CIAP, Roche) in a 100µl reaction containing 20U CIAP. The dephosphorylation reaction was stopped by heating to 68°C for 10 minutes in the presence of 15mM EDTA. The sample was then phenol : Chloroform extracted and ethanol precipitated before resuspending at 1µg/µl.

2.5.4 Ligation of Partially Digested OvHV-2 DNA to Cosmid Arms

A 2.5µg sample of partially digested genomic DNA was ligated to 1µg SuperCos 1MW DNA (Digested with *Xba*I, CIAP and *Bam*HI) in a 20µl reaction as described. New England Biolabs DNA ligase was used, as the ligase buffer does not contain polyethylene glycol, which can inhibit packaging.

2.5.5 Packaging the Ligated DNA

The ligated DNA was packaged into recombinant λ phage using a Gigapack III XL packaging kit (Stratagene) according to the manufacturer's instructions. The packaging extract (in a 25µl volume) was thawed on ice and the ligated DNA added immediately (1 - 4µl containing 0.1 - 1µg of ligated DNA). The tube was then stirred with a pipette to mix, before incubating for 2 hours at 22°C in a water bath. A 500µl volume of SM buffer (0.1M NaCl, 50mM Tris.HCl, 0.01% (w/v) gelatine (VWR)) was then added to the tube, followed by 20µl of chloroform. The sample was then spun briefly to sediment the debris and stored at 4°C for titration. A positive control

packaging reaction was also prepared using the packaging protocol as described but packaging 1µl of the λ c1857 wild-type λ control DNA provided with the packaging kit. This reaction was stopped using 1ml SM buffer.

2.5.6 Preparation of Bacteria For Titration of Packaging Reactions

The *E. coli* strain XL1-Blue MR (Stratagene) was used for titration of cosmid packaging reactions and the VCS257 strain provided with the packaging kit was used to titre the positive control packaging reaction. A single colony of the required bacteria was grown overnight in LB medium supplemented with 10mM $MgSO_4$ and 0.2% (w/v) maltose at 37°C with shaking. A 10µl sample of bacteria was inoculated into 10ml LB medium supplemented with 10mM $MgSO_4$ and 0.2% (w/v) maltose and grown for 4 - 6 hours at 37°C with shaking, to an OD_{600} of less than 1.0. The bacteria were then pelleted by centrifuging at 500 x *g* for 10 minutes and resuspended in half the original volume with sterile 10mM $MgSO_4$. The bacterial cells were then diluted to an OD_{600} of 0.5 in 10mM $MgSO_4$ and stored on ice until used.

2.5.7 Titration of the Positive Control Packaging Reaction

Serial dilutions of the control packaging reactions from 10^{-1} to 10^{-4} were prepared in SM buffer. A 10µl sample of the 10^{-4} dilution was then added to 200µl of the VCS257 host strain, diluted to an OD_{600} of 0.5 in 10mM $MgSO_4$. The mixture was incubated at 37°C for 15 minutes, before adding 3ml of LB top agar (LB medium with 0.7% agarose; Seakem), melted and cooled to 48°C. The dilution was then poured onto dry, pre warmed LB agar plates and the plates incubated overnight at 37°C. After incubation the plaques were counted, approximately 400 plaques being expected in the 10^{-4} dilution plate when the reaction was stopped with 1ml SM buffer.

2.5.8 Titration of the Cosmid Packaging Reaction

A 1:10 and a 1:50 dilution of the cosmid packaging reaction was prepared in SM buffer. A 25µl sample of each dilution was then mixed with 25µl XL1-Blue MR cells diluted to an OD_{600} of 0.5 in 10mM $MgSO_4$, and incubated at room temperature for

30 minutes. LB broth (200µl) was then added to each sample and the samples incubated for 1 hour at 37°C with shaking. The cells were then pelleted by centrifugation for 5 minutes at 5,000 x *g*, and the pellet resuspended in 50µl of fresh LB broth. The cells were then plated onto LB-Amp agar plates using a sterile spreader and the plates incubated overnight at 37°C. The colonies grown were counted and the titre calculated in colony forming units (cfu)/ml.

2.5.9 Amplification of Cosmid Libraries

Packaged cosmid DNA (i.e., the remainder of the packaging reaction after titration) was mixed with an equal volume of XL1-Blue MR cells diluted to an OD_{600} of 0.5 in 10mM $MgSO_4$, and the mixture incubated at room temperature for 30 minutes. LB broth (4 volumes) was added and the tube incubated at 37°C for 1 hour with shaking. The cells were then harvested by centrifuging for 10 minutes at 500 x *g* and resuspended in 500µl LB broth, before spreading onto a 150mm LB-Amp agar plate. After incubating the plate overnight at 37°C, 3ml LB broth was poured onto the plate and the colonies scraped into the liquid using a sterile spreader. The broth was pipetted into a fresh tube and the plate washed with another 3 ml LB broth. Sterile glycerol was then added to a final concentration of 20%(v/v) and ampicillin (Sigma) to 50µg/ml. The amplified library was then mixed and aliquoted into several samples, which were stored at -80°C.

2.5.10 Titration of The Amplified Cosmid Library

Serial dilutions of the amplified cosmid library were made from 10^{-1} down to 10^{-6} in LB broth. A 50µl sample of each dilution was plated onto LB-Amp plates, and incubated overnight at 37°C. The titre in cfu/ml was then calculated.

2.5.11 Screening Amplified Cosmid Libraries

The amplified cosmid library was plated onto a 150mm LB-Amp plate to give individual colonies (approximately 4,000 colonies). After incubation overnight at 37°C the colonies were transferred to a nylon membrane (Magnalift, Osmonics). The position of the membrane on the plate was marked, by pushing a needle through the membrane to the bottom of the plate and marking the position on the bottom of the

agar plate. The membrane was then lifted off and placed colony side up on a fresh 150mm LB-Amp agar plate. This plate and the original plate were incubated for 4 hours at 37°C. The membrane was then treated for 2 minutes in denaturation solution (0.5M NaOH), followed by neutralising solution (1MTris.HCl pH 7.4). The membrane was then soaked in neutralising solution and agitated in order to remove the bacterial debris, before UV crosslinking using the Stratalinker (Stratagene).

2.5.12 Generation of Probes For Library Screening

Probes were generated by PCR of known OvHV-2 sequence using either DNA extracted from OvHV-2 infected cell lines, or cosmid DNA as a template. Probes were generally 300-500bp in size. The PCR products were purified using the CONCERT PCR purification system as described (Section 2.4.8), before random primed labelling with $^{\alpha 32}$P dCTP.

2.5.13 Probing The Amplified Cosmid Library

Colonies of interest were detected by probing the membrane using $^{\alpha 32}$P dCTP labelled DNA probes. Membranes were pre-hybridised for 24h in 6 x SSC, 5 x Denhardt's reagent, 0.5% SDS, 100µg/ml denatured fragmented salmon sperm DNA at 65°C, before hybridising overnight at 65°C. Membranes were then washed as described for Southern blotting, covered in Saran wrap and exposed to x-ray film (Kodak X-Omat) at -70°C for 24 – 48 hours.

2.5.14 Low Stringency Hybridisation

The library was probed by low stringency hybridisation using Ultrahyb sensitive hybridisation buffer (Ambion) as described (Section 2.2.6), using a hybridisation and washing temperature of 37°C. Hybridisation probes were $^{\alpha 32}$PdCTP labelled as before.

2.5.15 Identification of Colonies of Interest

The needle holes on the membranes were marked on the overlying x-ray film using waterproof pen and the correct orientation of the x-ray film on the plate determined by aligning marks on the developed x-ray film to those on the original plate. Positive

colonies were then identified by the position of the x-ray film spots on the plate. Since a there were a large number of colonies on the original plate, it was not usually possible to identify individual positive colonies on the first screen, therefore the colonies round the positive area were scraped off the plate and resuspended in LB-Amp before re-plating to single colonies and re-screening to identify positive colonies. The positive colonies were picked and grown overnight at 37°C in 10ml LB-Amp broth and cosmid DNA for sequencing prepared from the samples using a QIA-spin mini prep kit (QIAGEN) as directed. Samples were then analysed by restriction enzyme digestion and agarose gel electrophoresis. Samples containing inserted DNA were then sequenced at each end of the insert. The sequence was then compared with sequence in the Genbank database.

2.5.16 DNA Sequencing

DNA sequencing was performed using the di-deoxy chain termination sequencing method by Mr Ian Bennet (Dept of Veterinary Pathology, University of Edinburgh). Reactions were performed using a SequiTherm EXCEL II DNA Sequencing kit (cycle sequencing protocol) on a 4000L automated sequencing machine (MWG-Biotech).

2.6 Sequence Analysis

2.6.1 Nucleotide Sequence Analysis

Analysis of sequenced DNA by comparison to database sequences was done using the basic alignment search tool (BLAST) programs on the NCBI web site http://www.ncbi.nlm.nih.gov/BLAST/ (Altschul *et al.*, 1990). Nucleotide sequences were compared to each other using the "pairwise blast" program. Analysis of sequence for the presence of open reading frames was performed using the NCBI ORF finder. Analysis of restriction sites and other features was done using the European Molecular Biology Open Software Suite (EMBOSS), provided by the UK Human Genome Mapping Project computer services, UK HGMP Resource Centre, Hinxton, Cambridge, CB10 1SB (http://www.hgmp.mrc.ac.uk/). Sequence was analysed for splice donor and acceptor sites using the program NetGene2 (Brunak *et*

al., 1991) and translational start and stop sites were predicted using HMMgene (Krogh, 1997). These tools were found at the Centre for Biological Sequence Analysis (CBS) prediction servers at http://www.cbs.dtu.dk.

2.6.2 Protein Sequence Analysis

Protein sequence database comparisons were done using the protein-protein BLAST (blastp) program at http://www.ncbi.nlm.nih.gov/blast/. Alignment of protein sequences for comparison was carried out using the ClustalW multiple sequence alignment program (Thompson *et al.*, 1994) and shaded diagrams of the sequence alignments carried out using the BOXSHADE program, both tools can be found at http://www.ch.embnet.org. Hydrophobicity plots of proteins were performed using the method of (Kyte & Doolittle, 1982), using the program ProtScale of the Expert Protein Analysis System (ExPaSy) web site at http://www.expasy.ch. Analysis of protein sequence for antigenic sites was carried out using the program ANTIGENIC, found on the JEMBOSS suite at http://www.hgmp.mrc.ac.uk/. This predicts antigenic sites using the method of (Kolaskar & Tongaonkar, 1990). Analysis of the sequence for transmembrane regions was done using the program TMHMM 2.0 (Sonnhammer *et al.*, 1998). Prediction of signal cleavage sites was done using the program SignalP (Nielsen *et al.*, 1997), analysis of the sequence for N-glycosylation sites was done using the program NetNGlyc 1.0, (Gupta *et al.*, 2002) and prediction of O-glycosylation sites was done using the NetOGlyc program (Hansen *et al.*, 1995). These tools were available at the Centre for Biological Sequence Analysis (CBS) prediction servers at http://www.cbs.dtu.dk. Analysis of the protein for features such as proline and serine rich regions was done using the ProSite tool, which scans a protein against the PROSITE database of protein families and domains (Falquet *et al.*, 2002), this was found at the Expert Protein Analysis System (ExPaSy) molecular biology server at http://www.expasy.ch.

2.7 RNA Analysis

2.7.1 Extraction of Cytoplasmic RNA from Cells

Cells (10^5 – 5 x 10^7) were washed twice in PBS before resuspension in 1ml ice cold Tris saline (25mM Tris pH 7.4, 130mM NaCl, 5mM KCl). The suspension was then centrifuged for 30s at 1000 x *g* and the pellet resuspended in 400μl Tris saline. A 100μl volume of Igepal buffer (1% Igepal (Sigma), 0.5% sodium deoxycholate, 0.01% dextran sulphate in Tris saline) was added and the sample mixed by gentle inversion. This resulted in lysis of the plasma membranes but not the nuclear membranes. The lysate was centrifuged at 2,000 x *g* for 1 minute and the nuclear pellet discarded. The supernatant was transferred to a new tube containing 500μl phenol : chloroform : iso-amyl alcohol (v/v), 25μl 20% SDS and 15μl 5M NaCl. The sample was then mixed by vortexing, followed by centrifugation at 10,000 x *g* for 2 minutes. This was repeated until the interface was clear (2 – 3 times) and the RNA was then extracted with chloroform : iso-amyl alcohol in order to remove any remaining phenol. The RNA was then precipitated by the addition of 1ml 96% (v/v) ethanol to the aqueous phase and incubation at –20 °C for 1 hour. The precipitate was then recovered by centrifugation at 4°C for 20 minutes at 10,000 x *g*. The sample was then washed in 80% ethanol, air dried and resuspended in 50μl sterile water. RNA was quantified using a Cecil spectrophotometer and the concentration calculated using the equation,

RNA concentration = Absorption at 260nm (A_{260}) x 40 x dilution factor

Where an A_{260} of 1.0 is equivalent to 40mg/ml of RNA. The RNA was also analysed by gel electrophoresis on a 0.8% agarose gel in 1 x TBE. RNA samples were concentrated if necessary by adding 1/10 volume of 3M Sodium Acetate pH5.2, 3 volumes of 95% Ethanol and 20mg glycogen (Ambion) and incubating at –20°C for at least one hour. The samples were then centrifuged at 10,000 x *g* at 4°C to pellet the RNA and resuspended in the appropriate volume of RNase free water.

2.7.2 Extraction of Total RNA From Cells

Total RNA was extracted from cultured cells using an RNeasy kit (QIAGEN). The cells (5×10^6 - 10^7) were harvested and pelleted by centrifugation for 5 minutes at 500 x *g*. The cells were then lysed by addition of 600µl of buffer RLT and the lysate homogenised by passing 10 times through a 20G needle attached to a 2ml syringe. A 600µl volume of 70% ethanol was then added and the sample added to an RNeasy spin column. After centrifugation the column was washed once in buffer RW1 and twice in buffer RPE and the RNA was eluted in 30-50µl RNase free water.

2.7.3 Extraction of Poly A RNA From Cells

This was performed using an Oligotex Direct mRNA purification kit (QIAGEN) according to the manufacturer's instructions. Cells ($5 \times 10^6 - 2 \times 10^7$) were harvested and pelleted by centrifugation for 5 minutes at 500 x *g*. The cells were lysed by addition of buffer OL1 (0.6ml) to which ß-mercaptoethanol had been added to a final concentration of 4.3M. The lysate was homogenised by passing 10 times through a 19G needle attached to a 2ml syringe. Buffer ODB (1.2ml) was then added and the sample mixed before the cell debris was removed by centrifuging for 3 minutes at 10,000 x *g*. The supernatant was transferred to a new tube and 110µl oligotex suspension added before vortexing and incubating at room temperature for 10 min. The oligotex was then pelleted by centrifugation for 5 minutes at 10,000 x *g* and the supernatant removed before resuspension of the oligotex and mRNA pellet in 100µl buffer OL1 by vortexing.

In order to further reduce ribosomal RNA, buffer ODB (400µl) was added and the sample incubated at 70°C for 3 minutes, followed by incubation for 10 minutes at room temperature. The oligotex and mRNA complex was then pelleted by centrifugation for 5 minutes at 10,000 x *g* and the pellet resuspended in 350µl buffer OW1 by vortexing. The sample was then pipetted onto a small spin column and centrifuged for 1 minute at 12,000 x *g*. The membrane was washed twice in 600µl buffer OW2 (10mM Tris-Cl pH7.5, 150mM NaCl, 1mM EDTA) and the column transferred to a new collection tube. A 100µl volume of buffer OEB (5mM Tris-Cl, pH 7.5) heated to 70°C was pipetted onto the spin column and the sample pipetted 3 or 4 times to resuspend the resin. The RNA was then eluted by centrifugation for 1

minute at 10,000 x *g* and the process repeated once. The mRNA was quantitated by spectrophotometry and concentrated by ethanol precipitation before resuspension in the appropriate volume of diH_2O.

2.7.4 Northern Analysis of RNA

Electrophoresis of RNA was carried out on 1.2% agarose gels containing 1 x MOPS buffer and 2.2M formaldehyde. Generally 5 – 10 µg of total RNA or 1 – 10 µg of polyadenylated RNA was loaded onto the gel alongside 5µg of an RNA molecular weight marker (0.24 - 9.5Kb RNA ladder, Invitrogen). RNA samples were suspended in 0.5 x MOPS, containing 50% formamide and 17.5% formaldehyde in a 20µl volume. Samples were then denatured by heating to 65°C for 15 minutes, followed by cooling on ice. A 2µl volume of formaldehyde gel loading buffer (50% glycerol, 1mM EDTA (pH8.0), 0.25% bromophenol blue) was added before loading. Electrophoresis was carried out overnight in 1 x MOPS buffer (20mM MOPS (pH 7.0), 8mM Sodium Acetate, 1mM EDTA (pH 8.0)) at 35V, during which the buffer re-circulated using a peristaltic pump. The gel was rinsed in distilled water and photographed before blotting overnight. Northern blots were set up in the same way as Southern blots except that transfer was carried out in 10 x SSPE. After transfer, the position of the wells was marked on the membrane and the RNA fixed by UV cross-linking using the Stratalinker (Stratagene). The lane containing the RNA marker was cut off and stained in the same way as for DNA ladders (Section 2.2.3).

2.7.5 Reverse Transcription of RNA

For reverse transcription 1-2 µg total RNA or 0.5-1µg polyadenylated RNA was used. RNA was first incubated in a 10µl total volume with 1U amplification grade DNaseI (Invitrogen) in 1 x DNaseI reaction buffer for 15 minutes at room temperature. The DNaseI was inactivated by adding 1µl of 25mM EDTA and heating for 10 minutes at 65°C. Whilst still hot, 0.5µg oligo dT primer or 250ng random primer (Invitrogen) and 1 µM each dATP, dGTP, dCTP, dTTP were added and the reaction mixed then cooled on ice. The reaction was then made up to a final concentration of 1 x first strand buffer (Invitrogen) and 0.2µM DTT and 40U recombinant ribonuclease inhibitor (RNase OUT, Invitrogen) was added before

incubation at 42°C for 2 minutes. Reverse transcriptase (200U) (Superscript II, Invitrogen) was then added, before incubation at 42°C for 50 minutes (if random primers were used, a 10 minute incubation at 25°C was required before the 50 minute incubation at 42°C). The enzyme was then inactivated by heating to 70°C for 10 minutes. A 2µl volume of the reaction was used as a template for PCR reactions.

2.8 Protein Analysis

Methods of protein analysis were performed based on methods described by Harlow & Lane, (1988).

2.8.1 Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analysis of proteins was performed using an Atto AE-6400 Dual Mini slab kit by the method of Laemmli, (1970). The glass plates were cleaned with 70% ethanol and the apparatus assembled as per the manufacturers instructions. The resolving gels were 12% (w/v) acrylamide (Ultra Pure Proto Gel, 30% (w/v) acrylamide:0.8% (w/v) bis-acrylamide, National diagnostics) 0.1% (w/v) SDS, 375mM Tris pH8.8, 0.04% N,N,N' Tetraethylmethylenediamine (TEMED, Sigma), and 0.1% (w/v) Ammonium persulphate (APS, Sigma). The resolving gel was poured and overlain with a small amount of overlay buffer (375mM Tris, pH8.8, 1% SDS (w/v)), then left to set. After the overlay buffer was removed from the surface of the solidified resolving gel, the stacking gel (125mM Tris, pH6.8, 0.1% (w/v) SDS, 5% (w/v) acrylamide, 0.1% (w/v) APS, 0.1% (v/v) TEMED) was poured over the top and a plastic comb inserted to form the wells. When set, the gels were assembled in the tank and SDS-PAGE running buffer (24mM Tris, 250mM glycine, 0.1% (w/v) SDS) added to the reservoirs. Samples for analysis were diluted 1:1 in 2 x SDS-PAGE sample buffer (100mM Tris (pH6.8), 4% (w/v) SDS, 4% (v/v) β-Mercaptoethanol, 20% (v/v) glycerol, 0.1% (v/v) bromophenol blue) and heated in a boiling water bath for 10 minutes before loading onto the gel. Samples of cells for analysis were usually resuspended in 100µl SDS-PAGE sample buffer per 10^6 cells. A sample of 10 – 20µl was then loaded onto the SDS-PAGE gel. The samples included a 5µl sample of broad range pre-stained SDS-PAGE standards (BioRad) in

order to estimate protein molecular weight relative to the standards. Electrophoresis was started at 80V until the dye front had reached the resolving gel then continued at 180V until the dye front had reached the bottom of the gel.

2.8.2 Coomassie Staining of SDS-PAGE Gels

SDS-PAGE gels were stained in Coomassie blue stain (0.25% (w/v) Coomassie blue, Sigma) 10% (v/v) glacial acetic acid, 50% (v/v) methanol) overnight with gentle shaking and de-stained over a period of 4 – 8 hours in a few volumes of Coomassie de-stain (50% (v/v) methanol, 10% (v/v) acetic acid) until excess stain had been removed. The gel was then dried onto 3MM paper using a model 583 gel dryer and HydroTech vacuum pump (BioRad).

2.8.3 Transfer of Proteins to Nylon Membranes (Western Blotting)

After SDS-PAGE, proteins were transferred to a PVDF membrane (Immobilon-P, Millipore) by the tank wet transfer system using a Mini Trans Blot Cell (BioRad). The apparatus was assembled according to the manufacturer's instructions and the tank filled with transfer buffer (25mM Tris, 250mM Glycine, 20% (v/v) methanol) and transfer carried out at 250mA for 1-1/2 hours.

2.8.4 Immunoblotting

Membranes were incubated in blocking buffer (5% (w/v) non-fat skimmed milk in TBS) overnight at 4°C in order to minimise non-specific antibody binding. When sheep serum was used as a primary antibody, 5% (v/v) donkey or horse serum was included in the blocking buffer. Membranes were then incubated with primary antibody diluted in blocking buffer (antibodies used and concentrations are shown in table 2.1) for 2 hours with gentle agitation. The membrane was then washed 4 times in TBS with 0.2% (v/v) Tween-20 before incubation with the secondary antibody diluted in blocking buffer for 1 hour with gentle agitation. The membrane was then washed 4 times in TBS with 0.2% (v/v) Tween-20 as before. If necessary, this was followed by incubation with a tertiary streptavidin conjugate for 1 hour. The membrane was then washed 5 times in TBS with 0.2% (v/v) Tween-20 and once in TBS before detection of antibody binding.

Antigen	Primary	Secondary	Tertiary
OvHV-2 O8	Sheep sera 1: 10 to 1: 50	Biotinylated donkey anti-sheep Ig Sigma 1:10,000	Streptavidin- AP conjugate 1:5,000 Roche
GST	Goat anti GST 1: 500 Amersham		
HA	Monoclonal rat anti-HA (3F10) Roche 1: 2,000	Biotinylated rabbit anti rat Ig DAKO 1: 10,000	Streptavidin- AP conjugate 1:5,000 or Streptavidin- HRP conjugate 1:5,000 Roche
GST	Polyclonal rabbit anti GST 1: 500	Porcine anti rabbit alkaline phosphatase DAKO 1:1000	Not required
OvHV-2 O8	Rabbit sera 1: 100 to 1: 2000	Porcine anti rabbit alkaline phosphatase DAKO 1: 500 to 1: 2,000	

Table 2.1 Antibodies used in immunoblotting analysis. Concentrations used are shown in blue and suppliers are shown in red.

The tertiary streptavidin was conjugated to either alkaline phosphatase (AP) or horseradish peroxidase (HRP). In both cases, detection of the enzyme activity was used to detect antibody binding. Alkaline phosphatase activity was detected by incubation of the membrane with BCIP/NBT solution (Sigma FAST 5-bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium). Horseradish peroxidase activity was detected using the ECL Western Blotting Analysis System (Amersham Biosciences) according to the manufacturer's instructions. An equal volume of detection solution 1 was mixed with detection solution 2 and the membrane incubated with the mixture for 1 minute. Excess solution was then drained from the membrane. The membrane was then wrapped in Saran wrap and exposed to autoradiography film (Hyperfilm ECL, Amersham Biosciences) at room temperature for 15s to one hour, depending on the strength of the signal.

2.9 Antibody production

2.9.1 Production of a GST Fusion Protein

A 621 bp fragment of A8 was amplified by PCR of DNA extracted from OvHV-2 infected cell lines using the PCR primers O8pGEX-5' and O8pGEX-3' (Appendix 2). The PCR product was then purified and digested using *Bam*HI and *Eco*RI. After purification using the CONCERT PCR purification system the PCR product was ligated into the vector pGEX-3X (Amersham Biosciences) in frame with the *Schistosoma japonicum* glutathione S-transferase gene (GST), using the *Eco*RI and *Bam*HI sites. The construct was transformed into XL-1 Blue strain of *E. coli* (Stratagene) as previously described. Bacterial colonies were checked for the O8 construct inserted into pGEX-3X by "mini prepping" and restriction analysis of the plasmid DNA with *Eco*RI and *Bam*HI. Positive samples were tested for expression of the GST fusion protein. Individual colonies were picked and grown overnight at 37°C in 10ml LB broth with 100μg/ml ampicillin. The culture was then diluted 1 in 10 in fresh LB and ampicillin and grown to an OD_{595} of 0.6. Protein expression was then induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG, Invitrogen) to a final concentration of 0.1mM and the culture grown for a further 3 hours. A

1.5ml sample of the culture was then pelleted at 500 x *g* for 5 minutes and the pellet resuspended in 300µl PBS containing 1% Triton-X 100 and 1% Apoprotinin. The bacteria were then lysed by sonication on ice and the insoluble material pelleted by centrifugation at 2,000 x *g* for 5 minutes. The soluble fraction was then incubated with 50µl of a 50% suspension of equilibrated glutathione-sepharose beads for 5 minutes at room temperature and the beads then washed in PBS containing 1% Triton X-100 before pelleting at 2,000 x *g* for 30 seconds. The beads were then washed twice in PBS before pelleting and resuspending in SDS-PAGE sample buffer. The insoluble and soluble fractions of the bacteria were then tested for protein expression by SDS-PAGE analysis.

2.9.2 Isolation of Bacterial Proteins by Inclusion Body Preparation

Bacterial colonies were grown overnight and induced with IPTG as described (Section 2.9.1). The bacteria were then pelleted at 7,000 x *g* for 5 minutes and resuspended in inclusion body solution (100mM NaCl, 1mM EDTA, 50mM Tris (pH8.0)) to a final concentration of 10% (v/v). Lysozyme was added to 10mg/ml and the solution incubated at room temperature for 20 minutes. The sample was then centrifuged at 5,000 x *g* for 10 minutes, the supernatant discarded and the pellet transferred to ice. The pellet was then resuspended in inclusion body solution with 0.1% (w/v) sodium deoxycholate and incubated on ice for 10 minutes. Magnesium chloride was added to a final concentration of 8mM and DNaseI (Roche) to a final concentration of 10µg/ml and the sample was incubated at 4°C for 1 hour with occasional mixing. The inclusion bodies were then removed from solution by centrifuging at 6,000 x *g* for 10 minutes and the pellet washed once in inclusion body solution in 1% (v/v) NP40 and once in inclusion body prep solution before resuspension in 2 x SDS-PAGE sample buffer. The soluble and insoluble fractions were analysed by SDS-PAGE.

2.9.3 Immunisation of Rabbits For Antibody Production

Two three month old New Zealand White rabbits were injected subcutaneously between 4 sites with a total of 250µl of a 50% (v/v) solution of inclusion body preparation diluted in PBS, emulsified with an equal volume of Freund's complete

adjuvant (Difco). This was then repeated in two months time, except that inclusion body preparation was this time emulsified with Freund's incomplete adjuvant. Blood samples were taken before immunisation and 2 weeks after the second injection. The serum was then analysed for the presence of specific antibodies.

2.10 Analysis of Eukaryotic Cells

2.10.1 OvHV-2 Infected Cell Lines

All OvHV-2 infected cell lines for analysis were cultured at the Moredun Research Institute by Dr Hugh Reid and Ms Irene Pow. The bovine cell line BJ/1035 was cultured from the tissues of a natural case of MCF (Reid *et al.*, 1989). The OvHV-2 infected rabbit cell lines BJ/2222, BJ/2223, BJ/880 and BJ/2476 were cultured from the tissues of rabbits experimentally infected with SA-MCF (Reid *et al.*, 1983).

2.10.2 Fixation of Cells

Cells were harvested and resuspended in PBS at 1 x 10^6/ml. The cells were applied to glass microscope slides (Twinfrost slides, VWR) by cytocentrifuging using a cytospin 2 (ThermoShandon). The cells were then air dried before fixing either in 4% (w/v) paraformaldehyde in PBS for 10 minutes at room temperature, or for 5 minutes in 100% acetone at –20°C. Acetone fixed slides were air dried and stored at –20°C. After fixing in paraformaldehyde the slides were washed twice in PBS, then permeablised by incubation for 5-10 minutes in PBS containing 0.2% (v/v) Triton X-100. The slides were then washed four times with PBS, air dried and stored at -20°C.

2.10.3 Immunostaining of Fixed Cells

Fixed cells on slides were incubated in PBS with 5% (v/v) normal goat or pig serum (the serum used was from the species of animal that the secondary antibody was raised in) for 30 minutes at room temperature in order to minimise non-specific binding of the antibodies used. The slides were then incubated with the primary antibody diluted in PBS with 5% (v/v) normal serum for 2 hours at 37°C (antibodies used and concentrations are shown in table 2.2). Negative control slides were

Antigen	Primary	Secondary
OvHV-2 O8	Rabbit sera 1: 50 to 1: 250	Porcine anti rabbit FITC conjugate 1: 50 DAKO
HA	Rat monoclonal anti HA (3F10) 1: 250 Roche	Goat polyclonal anti rat Ig FITC conjugate 1: 250 Pharmingen

Table 2.2 Antibodies used in immunostaining. Concentrations used are shown in blue, and suppliers are shown in red.

incubated for 2 hours in PBS with 5% (v/v) normal serum. The slides were then washed 4 times in PBS containing 0.05% (v/v) Tween-20, before incubation with the secondary antibody diluted in PBS containing 5% (v/v) normal goat serum for 45 minutes at 37°C. The cells were then washed four times in PBS containing 0.05% (v/v) Tween-20, before drying in air and mounting in fluorescent mounting medium.

2.10.4 Fluorescent Microscopy

Samples for fluorescent microscopy were mounted in fluorescent mounting medium. (DAKO) and examined using a Nikon Diaphot 200 Ultraviolet Microscope.

2.10.5 Separation of Lymphocytes From Whole Blood

Heparinised rabbit blood was obtained from the Moredun Research Institute and the lymphocytes separated by Ficoll density centrifugation using Lymphoprep Solution (Nycomed). The blood was very slowly and gently pipetted onto an equal volume of Lymphoprep solution, so that the layers did not mix. The blood and Lymphoprep was then centrifuged for 20 minutes at 400 x *g*. The lymphocytes were then carefully removed from the interface between the serum and the Lymphoprep using a Pasteur pipette and were washed three times in Roswell Park Memorial Institute 1640 (RPMI 1640, Invitrogen) complete medium (RPMI 1640 with 10% FCS, 2mM L-glutamine, 70μg/ml penicillin, 10μg/ml streptomycin, 50μM ß-mercaptoethanol). The lymphocytes were then counted using a haemocytometer and resuspended at the required concentration.

2.10.6 Stimulation of Lymphocytes Into Activated Lymphoblasts Using Phytohaemagglutinin (PHA)

Lymphocytes were separated from blood using Lymphoprep as described (Section 2.10.4). The lymphocytes were then resuspended in RPMI complete medium at 2 x 10^6/ml and phytohaemagglutinin was then added to 7.5μg/ml. The lymphocytes were then incubated in a well of a 6 well plate such that the lymphocytes had adequate contact with each other, at 37°C in a humidified atmosphere containing 5% CO_2. After 48 hours, the lymphoblasts were harvested.

2.10.7 Mammalian Cell Culture

All cell lines were cultured in sterile plastic ware (Nunc) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. HEK 293 cells (Graham *et al.*, 1977) and MDBK cells (Madin, 1958) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% (v/v) foetal calf serum (FCS, Sigma), 70μg/ml penicillin (VWR), 10μg/ml streptomycin (Sigma) and 2mM L-Glutamine (VWR). A20 cells (Kim *et al.*, 1979) were grown in RPMI 1640 supplemented with 5% FCS, 70μg/ml penicillin, 10μg/ml streptomycin, 50μM ß-mercaptoethanol (Sigma) and 2mM L-glutamine.

2.10.8 Harvesting Cultured Cells

Adherent cells were harvested and passaged by removing the monolayer with trypsin. The medium was poured off and the monolayer washed in 0.02% versene (Invitrogen) before incubation with 0.25% trypsin (Invitrogen) until the monolayer could be removed by tapping the flask. The trypsin was then diluted with an equal volume of medium and the cells centrifuged for 5 minutes at 450 x *g*. The cell pellet was resuspended in medium and a 50μl aliquot mixed with an equal volume of 0.1% (w/v) trypan blue (Sigma). The cells were then counted and non-viable cells identified using a haemocytometer.

2.10.9 Transfection of Cultured Cells by Electroporation

Cells at 50% confluency were harvested, counted and resuspended at 2.5 x 10^6/ml. An 800μl sample of the cells was added to a 4mm electroporation cuvette (Equibio), 5 – 20 μg DNA added and the sample mixed. The cells were then electroporated using an Easyject electroporator (Equibio). HEK293 and A20 cells were electroporated using a double pulse protocol (600V, 25μF, 99mΩ followed by 0.1s delay then 250V, 1050μF, 99mΩ). MDBK cells were transfected using a single pulse protocol (270V, 2500μF, infinite resistance). A20 cells were transfected using either the double pulse protocol described, or a single pulse protocol (260V, 1800μF, infinite resistance). Immediately after electroporation, the cell suspension was removed from the cuvette into 10ml fresh medium and the medium divided between 2 wells of a 6 well plate. Transiently transfected cells were harvested 48 hours post

transfection. The transfection efficiency was estimated by transfecting the control plasmid pEGFPC1 (Clontech), which expresses GFP and examining cells under UV light.

Appendix 1 - Buffer and Stock solutions

TE buffer	Tris-HCl (pH8.0) 1mM EDTA
TAE buffer	0.04M Tris-Acetate 1mM EDTA
TBE buffer	450mM Tris-Borate 1mM EDTA
Luria-Bertani (LB) medium	1% (w/v) tryptone 0.5% (w/v) yeast extract 1% (w/v) NaCl
LB agar	LB containing 1.5% (w/v) bacto-agar
SOC medium	LB containing 20mM glucose, 20mM $MgCl_2$
20 x SSC	3M NaCl, 300mM Na Citrate, pH7.0
20 x SSPE	3M NaCl, 0.2M $NaH_2PO_4.H_2O$ 20mM EDTA pH7.4
Tris-buffered saline (TBS)	50mM Tris-HCl, 150mM NaCl, pH7.6
Phosphate-buffered saline (PBS)	150mM NaCl, 2.5mM KCl, 10mM $Na_2H_2PO_4$, 1mM KH_2PO_4, pH 7.4
50 x Denhardt's solution	1% (w/v) Ficoll 400, 1%(w/v) Polyvinylpyrrolidone, 1% (w/v) BSA (Fraction V)

Appendix 2 - PCR Primers

Primer	Primer pair sequence	Annealing Temp	Amplified region
O75-5' O75-3'	5' – GCTTCAGCTTACTCCCTTTAC – 3' 5' – TCAATCAGGTCCAGGTTTACA – 3'	53°C	OvHV-2 ORF 75 540bp
O57-5' O57-3'	5' – CAGCCTTTTGCTCACTGG – 3' 5' – CCATGTAGCAGAGATTGTC – 3'	52°C	OvHV-2 ORF 57 483bp
C75R-5' C75R-3'	5' – CTATTCTCCATCACACAC – 3' 5' – CAATTGAGTCTTGTCTCC – 3'	48°C	R-hand end cosmid C75 516bp
O33-5' O33-3'	5'- TAGGCAAATATGGCCACTAG – 3' 5' – GAAATGTTCCCAGGTATAGG – 3'	53°c	OvHV-2 ORF 33 385bp
O11-5' O11-3'	5' – CATCACTACCCTGGTGCTTG – 3' 5' – AGAGAATAGTGGTAGTCCTCG – 3'	57°C	OvHV-2 ORF 11 307bp
gB-5' gB-3'	5' – GGGCCTTTATCTAACGTATGAGA – 3' 5' – TCACAATGCAAACACTTAYCTGAGTAA – 3'	60°C	OvHV-2 gB 2.8kb
O2-5' O2-3'	5' – GGACGTACAGGTCTTATTAC – 3' 5' – GGTATGGTGCTGCCTAATC – 3'	53°C	OvHV-2 O2 387bp
O8pGEX -5' O8pGEX -3'	*Bam*HI 5' – GGGATCCCC TCTAAGTCCTCCATCTTTCTG – 3' *Eco*RI 5' – TGAATTCCCAGAAACAGTAACTGTGATCGG – 3'	51°C	OvHV-2 O8 621bp
O7/8-5' O7/8-3'	*Eco*RI 5' – GCGAATTCTTACTCGGTTAAACACAGGAC – 3' *Bam*HI 5' – CGGGATCCCTCCCAGTCACCATGAGG – 5'	55°C	OvHV-2 O7 – O8 3kb
O7/8 HA-5' O7/8 HA-3'	*Not*I 5' – GCGCGGCCGCTCTTCTCTCCCAGTCACCAT G – 3' *Bam*HI Stop Triple HA epitope 5' – GCGGATCCTTA AGCGTAGTCTGGAACGTCGTA TGGGTA AGCGTAGTCTGGAACGTCGTATGGGTA AGCGTAGTCTGGAACGTCGTATGGGTA CTCGGTTAAACACCACAGGACAATG - 3'	58°C	OvHV-2 O7- O8 triple HA-tagged 3kb

Primer	Primer sequence	Annealing temp	Amplified Region
O7-3'	5' – GGTAGCTGAACTCCACAC – 3'	55°C	O7 – used with O7/8-5' 228bp
O7/8spl	5' – CACTAGCTGTAGGTGGTG – 3'	55°C	O7 – O8 used with O7/8-5' 702bp
O8end-5'	5' – GCTGAGACACTCACTCAGC – 3'	55°C	End O8 – used with O7/8-3' 447bp
O8mid-5' O8mid-3'	5' – GTTGTGGTCGTGGTTGTTATG – 3' 5' – CAGTTAGTTCCGACCTAGAAG – 3'	56°C	Middle O8 881bp
Rgap-5' Rgap-3'	5' – CCTTCATTGACCTCAACTACAT – 3' 5' – CCAAAGTTGTCATGGATGACC – 3'	55°C	Rabbit GAPDH 400bp
C75Rlong Spl3long	5' – CGGCTCTCACGGAAAGAACTAGAAG – 3' 5' – AGTAGCACTGAGGTTTAACACGCTC – 3'	69°C	Across viral terminal repeats (TRs)
C75RlongI Spl3longI	5' – GGGGATTGGAAAGTAAACGTCG – 3' 5' – GTGCTACTATGAAGCCTTTATGC – 3'	61°C	TR Internal primers
Hgap-5' Hgap-3'	5' – TGGATATTGTTGCCATCAATGACC – 3' 5' – GATGGCATGGACTGTGGTCATG – 3'	55°C	Human GAPDH 400bp

Appendix 3 - Primers Used In Splinkerette PCR

In first round PCR, the primer pair was the splinkerette primer (splink primer) with a gene specific primer (GSP). In the second round of PCR, the primer pair was the internal splinkerette primer (splink.Int) with an internal gene specific primer (GSP. Int).

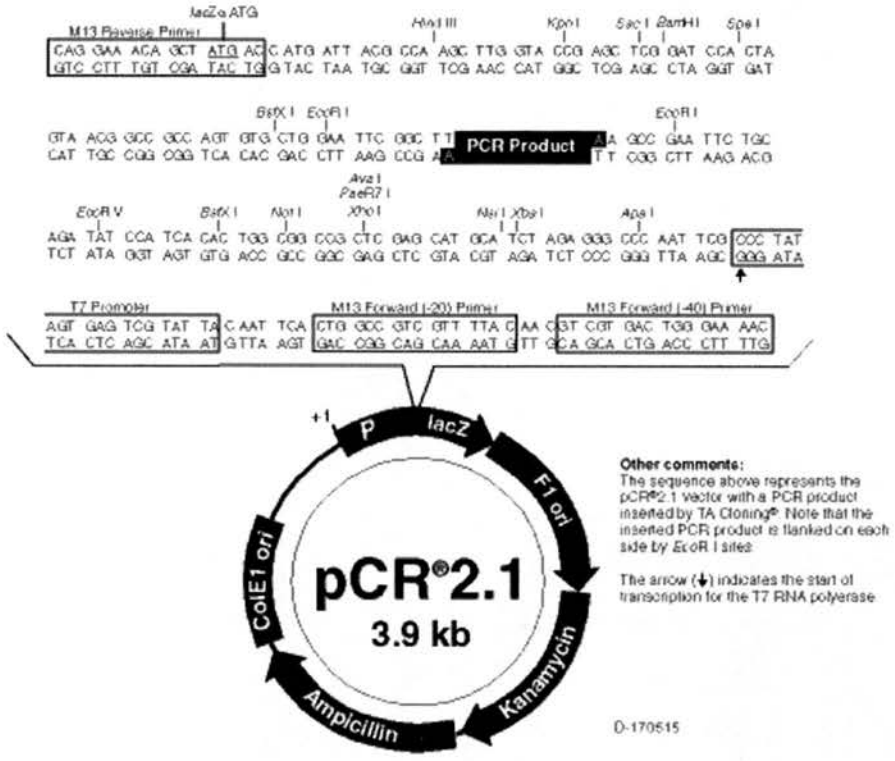
PRIMER	PRIMER SEQUENCE	Ann Temp
Splink primer	5' – CGAATCGTAACCGTTCGTACGAGAA – 3'	69°C
O2.GSP	5' – TCAAGAAGAGGTTACAGGAGCACGAG – 3'	73°C
Spl2.GSP	5' – GATGCTGAACACCATTAGGTGAGTG – 3'	69°C
Spl3.GSP	5' – AGTAGCACTGAGGTTTAACACGCTC – 3'	69°C
C75R.GSP	5' – TGGGTGTGTGATGGAGAATAGTCTG – 3'	69°C
C75R2.GSP	5' – GCCGAGAGTGCCTTTCTTGATCTTC – 3'	71°C
Splink.Int	5' – TTCGTACGAGAATCGCTGTCCT – 3'	61°C
O2.GSP.Int	5' – GCAGCAGCAAGAACGAGTAAG – 3'	59°C
Spl2.GSP.Int	5' – TCATCAGTGTTGAAGGGGATGC – 3'	61°C
Spl3.GSP.Int	5' – GTGCTACTATGAAGCCTTTATGC – 3'	61°C
C75R. GSP.Int	5' – GAGAATAGTCTGAATATACTGACC – 3'	61°C
C75R2. GSP.Int	5' – CCCCTAACCTTTCATTTGCAGC – 3'	61°C

Appendix 4 - Oligonucleotides Used For Construction of Splinkerette Linkers

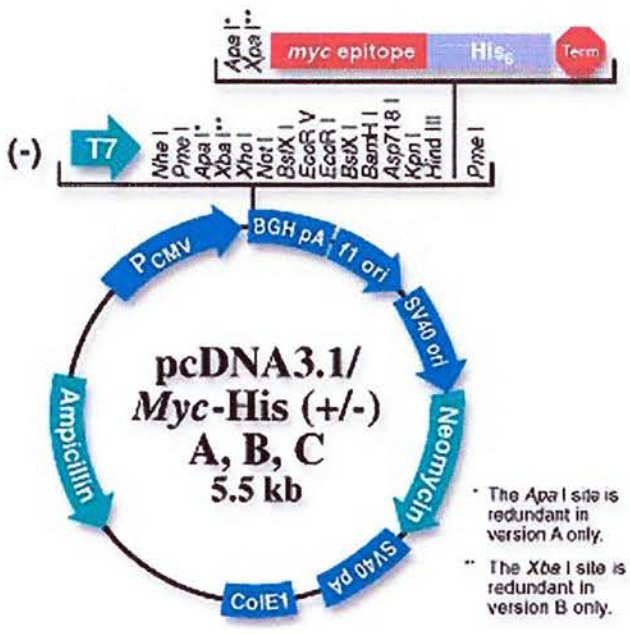
The splink Top oligo was used as the top strand in all linker molecules except the *Bam*HI linker which needed a top strand differing in one base, shown in pink.

NAME	SEQUENCE
Splink Top	5'– CGAATCGTAACCGTTCGTACGAGAATTCGTACG AGAATCGCTGTCCTCTCCAACGAGCCAAGA – 3'
Splink *Hin*dIII	5' – AGCTTCTTGGCTCGTTTTTTTTTGCAAAAA – 3'
Splink *Eco*RI	5' – AATTTCTTGGCTCGTTTTTTTTTGCAAAAA – 3'
Splink *Xba*I	5' – CTAGTCTTGGCTCGTTTTTTTTTGCAAAAA – 3'
Splink *Xma*I	5' – CCGGTCTTGGCTCGTTTTTTTTTGCAAAAA – 3'
Splink *Sal*I	5' – TCGATCTTGGCTCGTTTTTTTTTGCAAAAA – 3'
Splink *Bgl*II	5' – GATCTCTTGGCTCGTTTTTTTTTGCAAAAA – 3'
Splink *Bss*HII	5' – CGCGTCTTGGCTCGTTTTTTTTTGCAAAAA – 3'
Splink *Nhe*I	5' – CTAGTCTTGGCTCGTTTTTTTTTGCAAAAA – 3'
Splink Top *Bam*HI	5'– CGAATCGTAACCGTTCGTACGAGAATTCGTACG AGAATCGCTGTCCTCTCCAACGAGCCAAGG – 3'
Splink *Bam*HI	5' – GATCCCTTGGCTCGTTTTTTTTTGCAAAAA – 3'

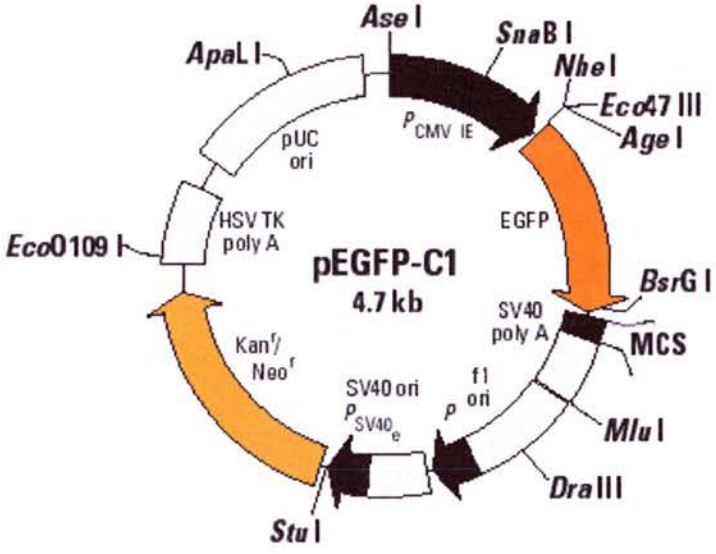
Appendix 5 – Cloning Vectors



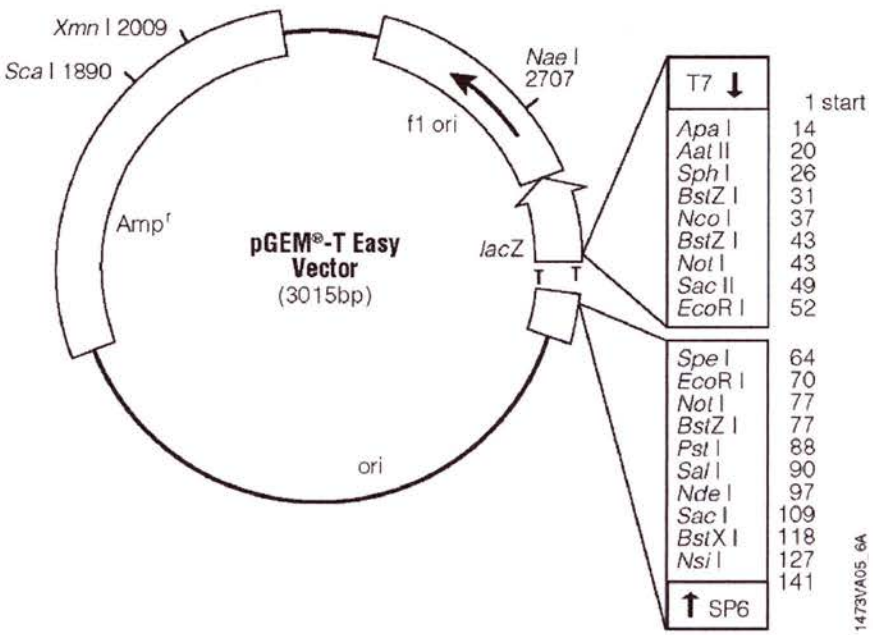
The vector pCR2.1 was obtained from Invitrogen



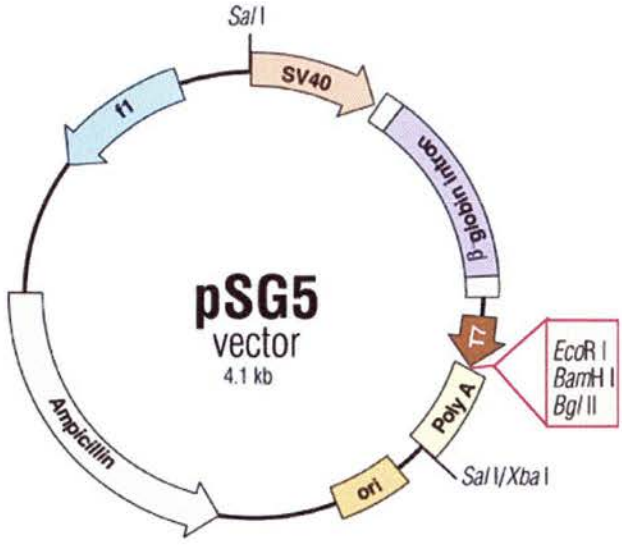
Plasmid pcDNA3.1/*Myc*-His, obtained from Invitrogen.



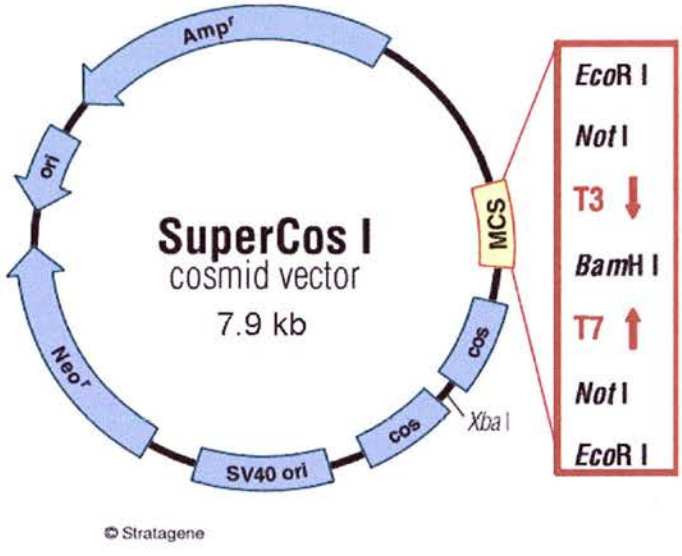
Plasmid pEGFP-C1, obtained from Clontech



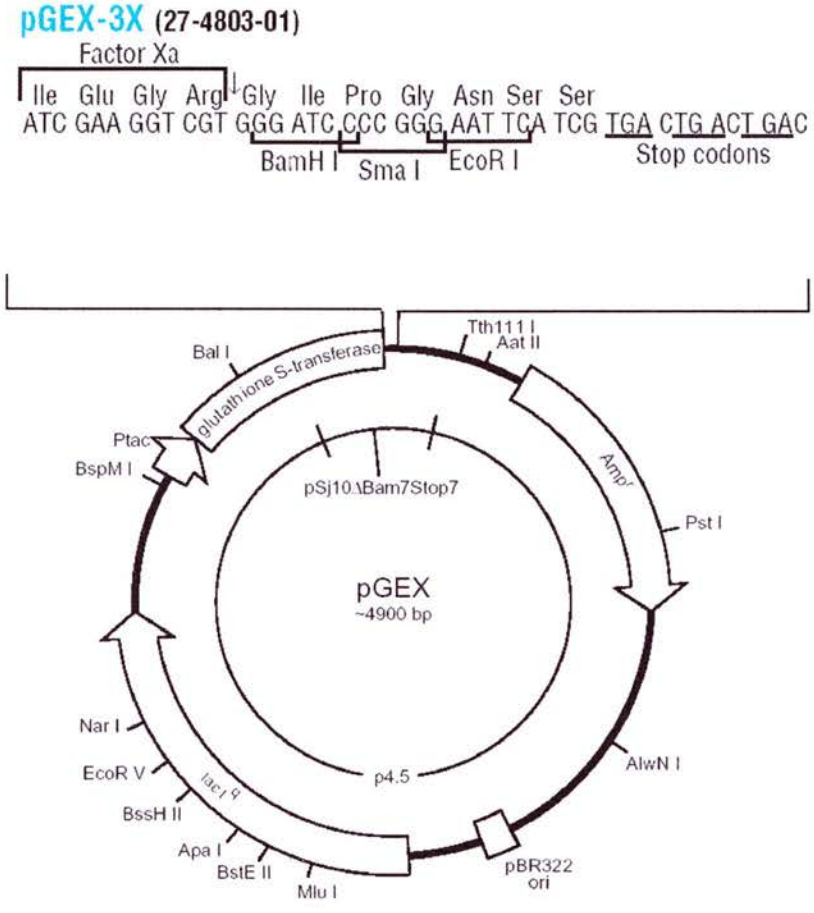
The vector pGEM – T Easy was obtained from Promega



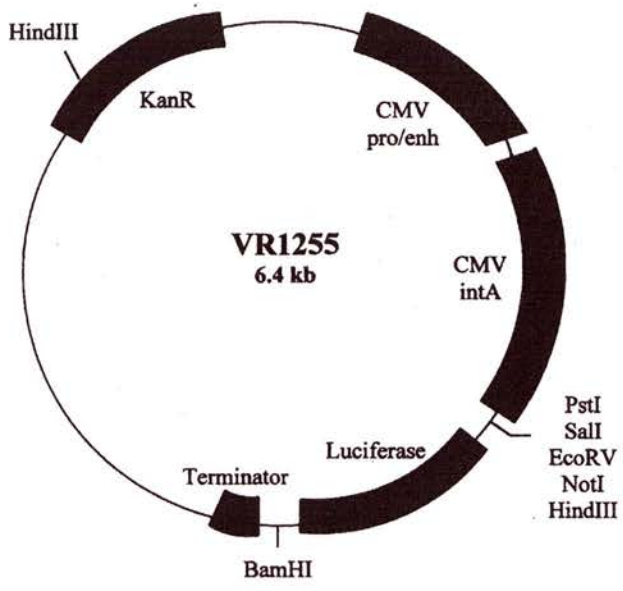
The control plasmids pSG5/Jκ-HA and pSG5/IRF7-HA were composed of either the Jκ-HA sequence or the IRF7-HA sequence cloned into the vector pSG5, and were provided by Dr Jeff Sample, St. Jude Children's Research Hospital, Memphis.



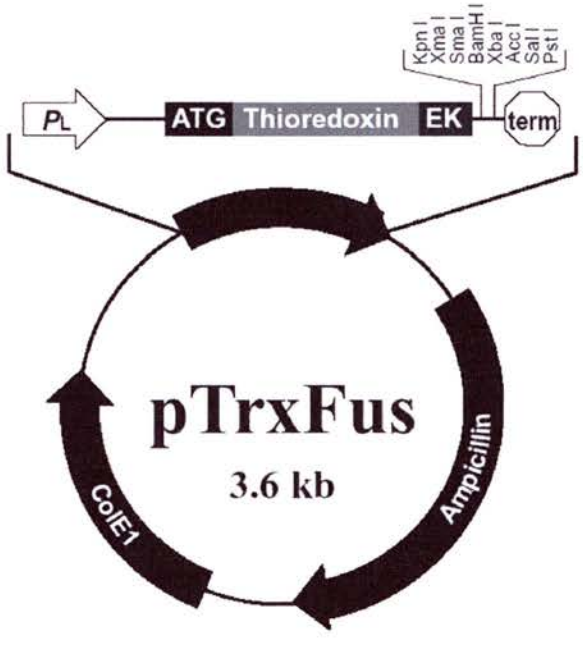
The cosmid vector SuperCos 1 was modified by the replacement of the *Not*I fragment of the vector cloning site with a cloning region with the sequence *Eco*RI – *Not*I – *Asc*I – *Pac*I – *Bam*HI – *Asc*I – *Pac*I - *Not*I – *Eco*RI to make SuperCos 1MW. The cosmid was kindly provided by Dr Andrew Davison, MRC Virology Unit, Glasgow.



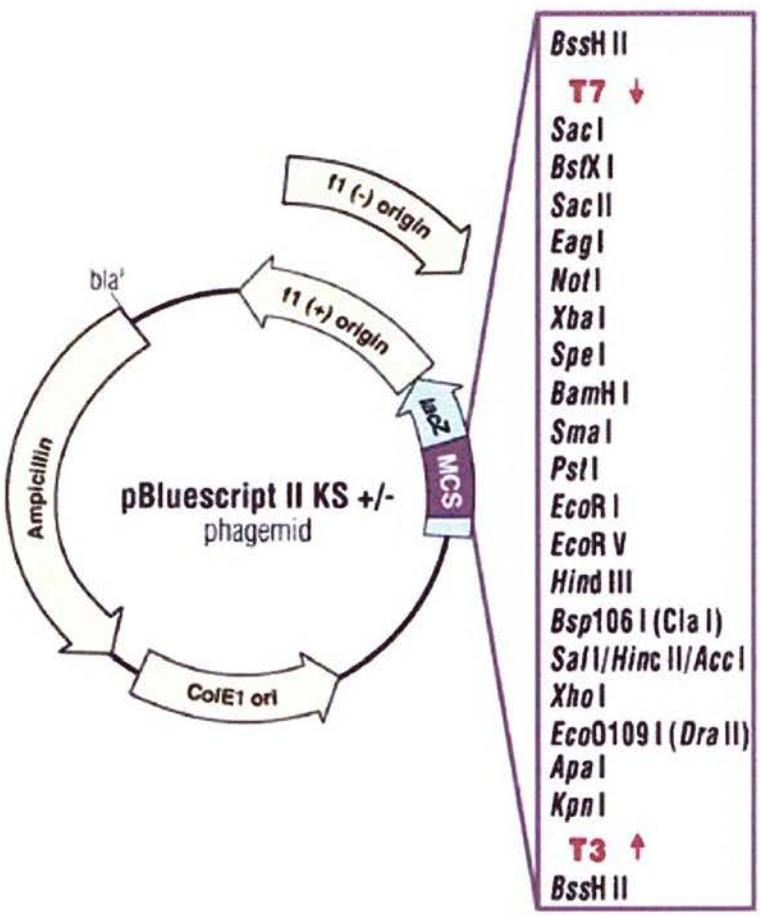
The vector pGEX-3X was obtained from Amersham Biosciences



The plasmid pVR1255 was obtained under license from Vical Incorporated.



The vector pTrxFus is obtained from Invitrogen. The OvHV-2 ORF 59 sequence was cloned into the vector pTrxFus using the *Pst*I and *Xba*I sites, resulting in the construct pTrxFus/ORF59 (Obtained from the Moredun Research Institute).



The vector pBluescript II KS was obtained from Stratagene

Appendix 6 - Commercial Suppliers

Ambion Inc, Ambion (Europe) Ltd, Ermine Business Park, Spitfire Close, Huntingdon, Cambridgeshire PE29 6XY
www.ambion.com

Amersham Biosciences UK Ltd, Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA HP7 9NA
www.amershambiosciences.com

Bio-Rad Laboratories Ltd, Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire HP2 7TD
www.bio-rad.com

Clontech - BD biosciences Clontech UK, 21 In Between Towns Road, Cowley, Oxford OX4 3LY
www.clontech.co.uk

DakoCytomaton Ltd, Denmark House, Angel Drove, Ely, Cambridgeshire CB7 4ET
www.dakocytomaton.com

EquiBio, Action Court, Ashford Rd, Middlesex. TW15 1XB
www.equibio.com

Flowgen, Findel House, Excelsior Rd, Ashby Park, Ashby de la Zouch, Leicestershire LE65 1NG
www.flowgen.co.uk

Invitrogen Ltd, 3 Fountain Dr, Inchinnan Business Park, Paisley PA4 9RF
www.invitrogen.com

Millipore, 80 Ashby Rd, Bedford, Massachusetts, USA.
www.millipore.com

MWG Biotech (UK) Ltd, Mill Court, Featherstone Rd, Wolverton Mill South, Milton Keynes MK12 5RD
www.mwgbiotech.com

Nalgenunc International, 75 Panorama Creek Drive, Rochester, NY 14625
www.nalgenunc.com

National diagnostics (UK) Ltd, Unit 4, Fleet Business Park, Itlings Lane, Hessle, East Riding of Yorkshire HU13 9LX
www.nationaldiagnostics.com

New England Biolabs (UK) Ltd 73 Knowl Piece, Wilbury Way, Hitchin, Hertfordshire, SG4 OTY
www.neb.com

Nycomed, Langebjerg1 P.O. Box 88 DK- 4000 Roskilde, Denmark
www.nycomed.com

Pharmingen – BD biosciences Pharmingen, 21 In Between Towns Road, Cowley, Oxford OX4 3LY
www.bdbiosciences.com/pharmingen/

Promega UK Ltd, Delta House, Chilworth Research Centre, Southampton SO16 7NS
www.promega.com

Roche Diagnostics Ltd, Bell lane, Lewes, East Sussex, BN7 1LG
www.roche.com

QIAGEN Ltd, Boundary Court, Gatwick Rd, Crawley, West Sussex RH10 9AX
www.QIAGEN.com

Sigma-Aldrich Company Ltd, Dorset, England
www.sigmaaldrich.com

Stratagene Europe, Gebouw California, Hogehilweg 15, 1101 CB Amsterdam, Zuidoost, The Netherlands
www.stratagene.com

ThermoHybaid, Action Court, Ashford Road, Ashford, Middlesex, TW15 1XB
www.thermohybaid.com

ThermoShandon, 171 Industry Drive, Pittsburgh, PA 15275 USA
www.shandon.com

Vical Incorporated, 9373 Towne Centre Drive, Suite 100, San Diego, California 92121-3088
www.vical.com

VWR international Ltd, Merck House, Poole, Dorset BH15 1TD
www.vwr.ltd.uk

UVP Ultra Violet Products Ltd, Unit 1, Trinity Hall Farm Estate, Nuffield Rd, Cambridge CB4 1TG
www.uvp.com

Chapter Three: Results

3.1 Replication of OvHV-2

3.2 Generation of OvHV-2 Genome Sequence

3.3 Characterisation of the O7 and O8 ORFs

3.1 Replication of Ovine Herpesvirus 2

Little is known about OvHV-2 viral gene expression in animals with SA-MCF, largely due to the fact that the lack of available sequence has meant that there are few reagents available for the study of OvHV-2 pathogenesis. From animals infected with Malignant Catarrhal Fever, Lymphoblastoid Cell Lines (LCL) can be propagated, some of which transmit disease (Reid *et al.*, 1989) (Reid *et al.*, 1983). Until this point, no evidence of virus particles or viral products have been found in the cell lines, although viral DNA homologous to that of AlHV-1 has been identified by hybridisation with AlHV-1 DNA (Bridgen & Reid, 1991). Since the OvHV-2 infected cell lines transmit disease, it is hypothesised that the complete OvHV-2 virus genome persists in the cell lines. The mode of viral persistence in the OvHV-2 infected cell lines is however, unknown.

The aim of this work was to further characterise the OvHV-2 infected cell lines, with respect to the mode of viral persistence in the cell lines and to quantify the amount of virus persisting in the cell lines. The ultimate aim of the work was to establish if there was evidence of viral replication in OvHV-2 infected cell lines and if possible, identify viral particles. This work was performed as a starting point to determine if the OvHV-2 infected cell lines were a suitable source of viral DNA from which the full OvHV-2 viral genome could be obtained.

3.1.1 Determination of the OvHV-2 Genome Copy Number

Initially it was decided to establish on average, how many viral genomes were present in each cell. The genome copy number was determined by a Southern blotting method. Serial dilutions of an OvHV-2 sequence (ORF59) were electrophoresed alongside digested DNA from 1.6 x 10^6 OvHV-2 infected LCLs. The intensity of any bands seen in the plasmid lanes of the blot was compared with that seen in the digested OvHV-2 DNA lanes after probing with an OvHV-2 specific probe.

In order to construct an OvHV-2 specific probe a clone containing OvHV-2 ORF 59 sequence (Coulter & Reid, 2002) cloned into the vector pTrxFus (Invitrogen) (Appendix 5) was obtained from the Moredun Research Institute. This

was named pTrxFus/ORF59. The OvHV-2 ORF 59 sequence was excised from this vector by digestion with *Xba*I and *Pst*I. The fragment was then gel purified by electroelution as described and labelled with α^{32}PdCTP.

3.1.1.1 Preparation of Dilutions of Plasmid DNA For Southern Blotting

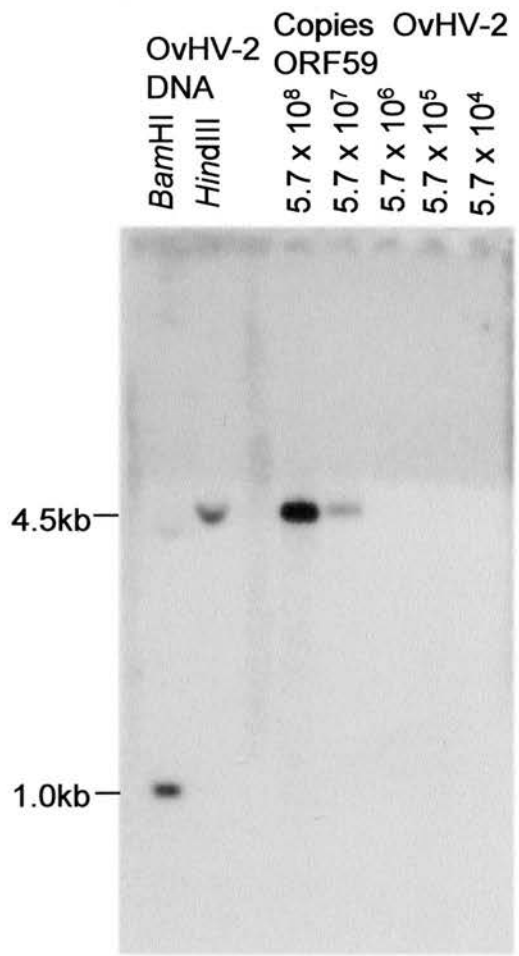
The plasmid TrxFus/ORF59 was 4.6kb in size. 1kb of DNA has an approximate molecular weight of 6.60 x 10^5 (Sambrook *et al.*, 1989) therefore a plasmid of 4.6kb has a molecular weight of 3.04 x 10^6. The weight of one mole of plasmid was therefore 3.04 x 10^6g. This meant that 1μg of plasmid contained 1.97 x 10^{11}molecules.

The aim was to make a dilution of plasmid DNA such that 5μl of the sample contained 10^9 molecules. Serial dilutions of this sample could then be made down to 10^5 molecules/5μl. A 5μl aliquot of each sample would then be linearised in preparation for electrophoresis. The pTrxFus/ORF59 plasmid stock was at 100ng/μl, therefore 5μl contained 500ng of DNA and 1 x 10^{11}molecules. In order to make a stock of 10^9 molecules/5μl, the plasmid stock was needed to be diluted 1/100 in TE pH8.0, however due to a mistake in the original calculations the plasmid dilution made was 1/175. Serial dilutions of this stock were then made in TE pH8.0, which were now from 5.7 x 10^8 molecules/5μl down to 5.7 x 10^4 molecules/5μl in 1 in 10 serial dilutions. A 5μl sample of each dilution was then digested in a 20μl reaction with *Pst*I, in order to linearise the plasmid. Gel loading buffer (4μl) was then added to each digested plasmid sample, before loading onto the gel.

3.1.1.2 Southern Analysis of the OvHV-2 DNA and ORF59 Plasmid Dilutions

DNA was extracted from the OvHV-2 infected bovine cell line BJ/1035 as described (Section 2.2.1). Genomic DNA extracted from OvHV-2 infected cell lines was termed OvHV-2 DNA. OvHV-2 DNA (10μg) was digested with *Bam*HI or *Hin*dIII in a 200μl reaction at 37°C. These enzymes were chosen because the GC content of OvHV-2 DNA was not known. Since the *Bam*HI restriction site is relatively GC rich, whereas the *Hin*dIII site is relatively AT rich, it was predicted that the DNA should digest well with one of these enzymes. After digestion, the samples were ethanol precipitated and resuspended in a 20μl volume of TE pH8.0. The OvHV-2 DNA

Figure 3.1.1 Southern Blot for Estimation of the Copy Number of OvHV-2 Genomes in Infected Cells



OvHV-2 DNA (10μg) extracted from the OvHV-2 bovine cell line BJ/1035 was digested with *Bam*HI and 10μg digested with *Hin*dIII. The digests were electrophoresed alongside serial dilutions of OvHV-2 ORF 59 DNA from 5.7 x 10^8 to 5.7 x 10^4. The electrophoresed DNA was analysed by Southern blotting using an OvHV-2 ORF 59 probe. The blot was exposed to film overnight and any bands compared to a 1kb DNA ladder.

samples were loaded onto a 1% agarose gel alongside the linearised plasmid samples and electrophoresed until the loading buffer dye front had migrated 10cm down the gel. The gel was then photographed to check for even loading and the DNA transferred to a nylon membrane (Magnagraph, Osmonics) by Southern blotting as described (Section 2.2). The membrane was probed using the $^{\alpha 32}$P labelled OvHV-2 ORF59 probe. After exposure of the blot to X-ray film the intensity of the resulting bands from the known copy numbers of plasmid DNA was compared with that of the two lanes of digested OvHV-2 DNA.

The *Bam*HI digest of the OvHV-2 DNA resulted in 2 bands at 1kb and 4kb approximately. This showed that there is a *Bam*HI site in the ORF59 DNA (Figure 3.1.1). The *Hin*dIII digest resulted in just one band at 4.5kb. The intensity if the bands of digested OvHV-2 DNA was similar to both the 5.7 x 10^7 and the 5.7 x 10^8 copies of plasmid lanes. The 10μg of genomic DNA was from 1.6 x 10^6 cells, therefore the OvHV-2 infected cells contain between 356 and 36 copies of viral genome. Since the intensity of the bands was more similar to the 10^8 copies of plasmid lane, it was estimated that the cells contain approximately 50 copies of viral DNA per cell.

3.1.2 Analysis of OvHV-2 Infected Cells by Gardella Gel Electrophoresis

It was not known in what form the viral DNA persisted in OvHV-2 infected cell lines. If the viral genomes were predominantly in the latent state, persistence was likely to be as a circular episome. If viral DNA replication was taking place, this would result in the production of linear viral DNA. In order to address this problem, Gardella gel electrophoresis was performed on OvHV-2 infected LCLs. A Gardella gel is a denaturing agarose gel whereby whole cells to be analysed are loaded onto wells in the denaturing section of the gel and gently lysed *in situ,* whilst undergoing slow electrophoresis (Gardella *et al.*, 1984). After lysis, the current applied to the gel is increased and the products of cell lysis are electrophoresed overnight. Circular DNA is retarded compared to linear DNA, therefore when the DNA is transferred to a nylon membrane and hybridised with an OvHV-2 specific probe, the presence of linear or circular viral DNA can be determined by comparison to controls.

Fresh OvHV-2 infected LCLs (2 x 10^6) were loaded onto the denaturing portion of the gel containing SDS and proteinase K as described (Section 2.3.2). Two

control cell lines were also analysed; the BCP-1 cell line is a KSHV infected B cell line (Boshoff *et al.*, 1998) in which approximately 1% of cells are lytically infected (Dr S.J.Talbot, Personal communication), whilst the remaining cells are latently infected. The S11 cell line is an MHV-68 infected B cell line (Usherwood *et al.*, 1996) in which up to 20-30% of cells can undergo productive cycle replication. The cells were subjected to electrophoresis slowly at 30V for 3 hours at 4°C though the denaturing gel, so that any episomal DNA would remain intact. Electrophoresis was then continued overnight at 110V.

After electrophoresis the DNA was transferred to a nylon membrane (Section 2.2), The position of the viral DNA on the gel was then determined by Southern blotting. OvHV-2 viral DNA was detected using a ^{32}P labelled OvHV-2 specific probe. This was made by PCR amplification of DNA extracted from the OvHV-2 infected cell line BJ/1350 using the primers O75-5' and O75-3' (Appendix 2). These primers were designed to amplify a 540bp region of OvHV-2 ORF 75. KSHV DNA was detected using a probe specific for the KSHV K1 gene (obtained from Dr. S.J. Talbot) and MHV-68 DNA was detected using a probe specific for the MHV-68 M2 gene (obtained from Dr A. MacRae). The OvHV-2 infected cell lines analysed were the bovine cell line BJ/1035 and the rabbit cell lines BJ/2222 and BJ/2223.

Hybridisation was initially carried out using 0.05 x BLOTTO in 6 x SSC, however no signal was detected. It was suspected that transfer of DNA onto the nylon membrane may not be optimal, therefore the Gardella gel was repeated and a control for DNA transfer was electrophoresed. This was 0.5μg cosmid A8 DNA (SuperCos 1MW containing the left-hand end of MHV-68, obtained from Dr A. MacRae). Hybridisation of the M2 probe to this DNA was detected by Southern blotting, however hybridisation of the virus specific probes to any of the viral DNA samples was not detected. The amount of DNA in the A8 control DNA sample, however, may have been quite large compared to the amount of viral DNA present in a latently infected cell line, therefore it was decided that a more sensitive detection system might be useful.

In order to increase sensitivity, Ultrahyb sensitive hybridisation buffer (Ambion) was used. This buffer contains 50% formamide and when detecting a high

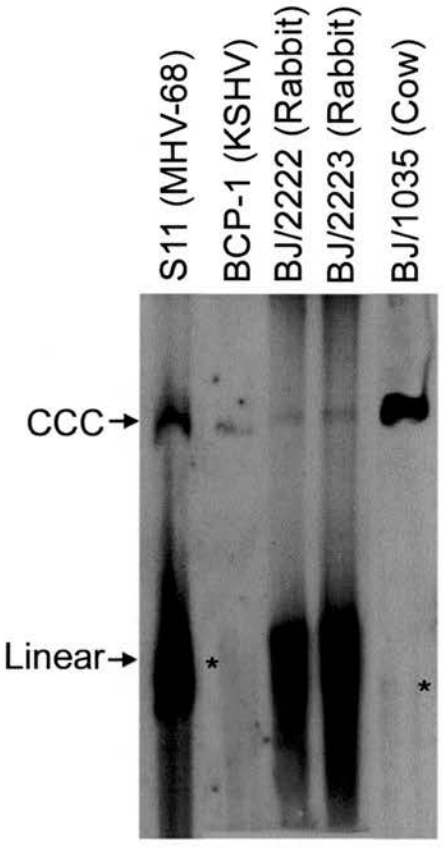
copy number target, only a 1-2 hour hybridisation is required. Lower abundancy targets such as the viral DNAs resolved by Gardella gel electrophoresis required overnight hybridisation. Hybridisation and washes were carried out at 42°C as described (Section 2.2.6). The blot was initially exposed to X-ray film for 2 days, after which a low intensity signal was detected for all samples. The membrane was therefore exposed for a further 2 weeks to increase the intensity of the signal (Figure 3.1.2). The S11 cell control showed an obvious upper band indicating episomal circular DNA and a heavy lower smeared band indicating linear viral DNA produced in viral DNA replication. The BCP-1 control cell line also showed two bands of viral DNA, indicating the presence of both linear and episomal DNA. The OvHV-2 infected rabbit cell lines (BJ/2222 and BJ/2223) both showed an obvious lower smeared band indicative of linear DNA, as would be produced in viral DNA replication. A faint upper band was also seen, indicating the presence of episomal circular viral DNA found in latently infected cells. In the OvHV-2 infected bovine cell line BJ/1035, the lower linear viral DNA band was of low intensity compared to that observed in the rabbit cell lines, whilst there was a strong upper band indicating episomal viral DNA.

The presence of retarded upper bands of viral DNA in the rabbit OvHV-2 infected cell lines and in the cattle cell lines was clear evidence that as suspected, all cell lines contained circular OvHV-2 viral DNA. This suggested that some cells in each cell line were latently infected with OvHV-2. The smeared high intensity lower bands seen in the rabbit cell lines were indicative of production of linear viral DNA, and therefore were evidence that the cell lines contained replicating viral DNA. The cattle cell line showed a barely detectable lower linear DNA band, which would seem to suggest that this cell line contained lower levels of viral DNA replication.

3.1.3 Analysis of RNA Extracted from OvHV-2 Infected Rabbit Cell Lines

Gardella gel analysis of OvHV-2 infected cells demonstrated evidence of viral DNA replication in rabbit cell lines. Since viral DNA replication and production of linear viral DNA is generally seen in the viral lytic cycle, the OvHV-2 infected cell lines were investigated for further evidence of lytic viral replication. The cell lines were

Figure 3.1.2 Gardella Gel Analysis of OvHV-2 Infected Cell Lines



Two OvHV-2 infected rabbit cell lines (BJ/2222 and BJ/2223) and one OvHV-2 infected bovine cell line (BJ/1035) were analysed by Gardella Gel Electrophoresis. The KSHV infected B-cell line BCP-1 and the MHV-86 infected B-cell line S11 were analysed as control cell lines. The gel was analysed by southern blotting and probed using ^{32}P labelled probes specific for OvHV-2 (ORF 75), KSHV (K1) and MHV-68 (M2). The position of covalently closed circular (CCC) and linear DNA are marked on the left side of the image. The stars mark bands which were visible on the x-ray film but did not reproduce well photographically.

therefore analysed for evidence of expression of productive cycle genes by northern analysis and RT-PCR.

3.1.3.1 Northern Analysis of OvHV-2 Infected Rabbit Cell Lines

Treatment of cell lines latently infected with EBV or KSHV with the phorbol ester TPA increases the proportion of cells lytically infected, therefore a sample of BJ/2222 OvHV-2 infected rabbit cells was incubated overnight with TPA at a concentration of 20ng/ml. Cytoplasmic RNA was extracted from the TPA treated OvHV-2 infected rabbit cells and from untreated cells as described (Section 2.7.1). A 5μg sample of total RNA was then analysed by northern blotting (Section 2.7.4). The blot was probed using an OvHV-2 ORF 57 probe. This was made by PCR amplification of OvHV-2 infected cell DNA using the primers O57-5' and O57-3' (Appendix 2). These primers amplified a 483bp region of OvHV-2 ORF 57. ORF 57 is an immediate early lytic gene and is thought to be involved in regulation of viral gene expression (Goodwin *et al.*, 2000). No signal was detected after exposure to film for 14 days therefore the ORF 57 probe was removed from the blot. Hybridisation of the blot was then repeated using an OvHV-2 ORF 75 probe. ORF 75 is a late gene encoding a putative tegument protein and it was thought that expression of this gene might be higher than that of ORF 57. No signal was detected however after a 14 day exposure to film, therefore it was concluded that levels of OvHV-2 mRNA in the BJ/2222 cells were low and treatment of the cells with TPA did not increase expression of viral genes to the level of detection. In order to increase the likelihood of detecting viral transcripts by northern blotting, analysis of poly A RNA would have been necessary however, at the time this was not possible due to the low availability of OvHV-2 infected cells.

3.1.3.2 RT-PCR analysis of OvHV-2 infected rabbit cell lines

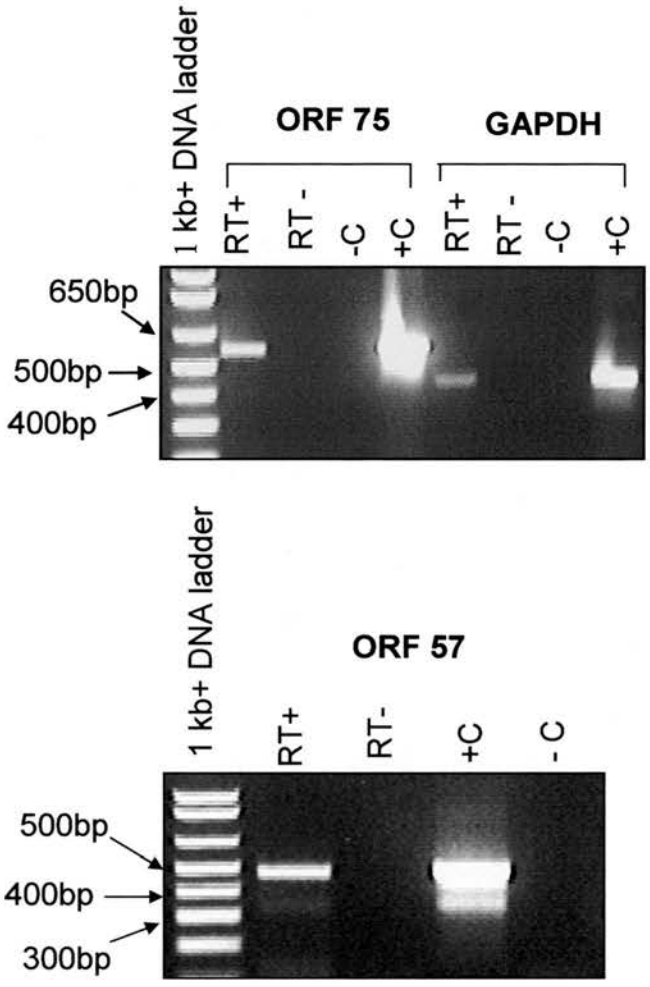
Northern analysis of RNA extracted from the OvHV-2 infected rabbit cell lines demonstrated that expression of viral transcripts was low, therefore in order to increase sensitivity of detection, samples of the same batch of RNA were analysed by RT-PCR. Two samples of RNA (1μg/reaction) were incubated with DNaseI as described and reverse transcribed using random primers (Section 2.7.5). For each

reaction a control sample was included, omitting reverse transcriptase enzyme from the reaction. This was to ensure any products were as a result of amplification of cDNA and not from amplification of contaminating genomic DNA.

Initially PCR amplification of the cDNA was performed using the primers Rgap-5' and Rgap-3' (Appendix 2), which were designed to amplify a 400bp region of the rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. This is a housekeeping gene that is expressed at substantial levels in all cells. In initial analyses, failure of the GAPDH PCR reaction indicated that the RT reactions had not worked. Phenol contamination of RNA is sometimes found in RNA samples extracted using methods involving the use of phenol. This can inhibit the action of reverse transcriptase enzymes, therefore a different method of RNA extraction was used. Total RNA was extracted from the BJ/2222 OvHV-2 infected rabbit cell line using a QIAGEN RNeasy kit (Section 2.7.2), as this does not involve the use of phenol.

PCR amplification of the new sample of total RNA using the GAPDH primers, resulted in product of the correct size of 400bp (Figure 3.1.3), therefore this confirmed that the reverse transcriptase reaction had worked. The cDNA was then used for PCR amplification reactions using the OvHV-2 ORF 75 primers O75-5' and O75-3' (Appendix 2) and using the OvHV-2 ORF 57 primers O57-5' and O57-3' (Appendix 2). The PCR products were analysed by gel electrophoresis. Products were detected from reactions using both the ORF 57 primer pair and the ORF 75 primer pair (Figure 3.1.3). This showed expression of both the OvHV-2 immediate-early gene ORF 57 and the OvHV-2 late gene ORF 75 in the rabbit OvHV-2 infected cell line BJ/2222. By comparison to other gammaherpesvirus genes, both ORF 57 and ORF 75 are expressed in the viral lytic cycle, therefore transcription of these genes in OvHV-2 infected rabbit cell lines provides evidence of early and late lytic viral gene expression in the cell lines.

Figure 3.1.3 RT-PCR Analysis of the OvHV-2 Infected Rabbit Cell Line BJ/2222



Total RNA was extracted from the OvHV-2 infected rabbit cell line BJ/2222. The RNA was used in reverse transcription reactions using random primers. The products of the RT reactions were amplified by PCR using the primers against rabbit GAPDH (Rgap-5' and Rgap-3'), OvHV-2 ORF75 (O75-5' and 3') and OvHV-2 ORF57 (O57-5' and 3') as indicated above the gel images. The products were analysed by agarose gel electrophoresis alongside a 1kb+ DNA ladder.

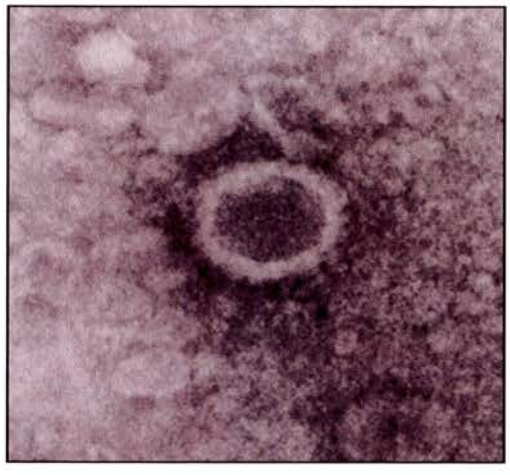
+C = PCR Positive control using OvHV-2 genomic DNA as template for ORF 75 and ORF 57 primers and MHV-68 cDNA template for GAPDH primers. –C = PCR negative control with dH_2O template. RT+ = RT-PCR product. RT– = RT-PCR control omitting reverse transcriptase enzyme.

3.1.4 Electron Microscopy of Rabbit Cell Line BJ/2222

Evidence of OvHV-2 viral DNA replication in the OvHV-2 infected rabbit cell lines was demonstrated by Gardella gel analysis and viral transcripts of the productive cycle genes ORF 57 and ORF 75 were detected by RT-PCR. To this point viral particles had not been observed in the OvHV-2 infected cell lines, however as a result of the evidence that viral replication occurred in these cell lines, the OvHV-2 infected cells were examined for the presence of viral particles. Sodium Butyrate treated and non-treated OvHV-2 infected rabbit cells were fixed in glutaraldehyde and examined by transmission electron microscopy, however no particles were visible after examination of a few hundred cells. An alternative method was therefore adopted based on that used by Arvanitakis *et al.*, (1996). Cells were lysed by freeze-thawing and the lysates pelleted by ultracentrifugation. The pellets were examined by transmission electron microscopy at Lasswade Veterinary Laboratory by Dr Martin Jeffrey and Ms Gillian McGovan. A herpesvirus particle was visualised (Figure 3.1.4), the icosahedral capsid typical of a herpesvirus was visible, however it was not obvious if the capsid was surrounded by a viral envelope.

In summary, it has been shown that there is evidence of productive cycle replication of OvHV-2 in infected rabbit cell lines, as shown by production of linear DNA as shown by Gardella gel analysis, production of mRNA for lytic cycle genes, and production of viral capsids, however the presence of fully enveloped viral capsids has not yet been shown.

Figure 3.1.4 An Electron Micrograph Obtained From Examination of Lysates of OvHV-2 infected Rabbit Cell line BJ/2222



Cells of the OvHV-2 infected rabbit cell line BJ/2222 were lysed by freeze thawing and concentrated by centrifugation as described in (Arvanitakis *et al.*, 1996). The fixed cell pellet was negatively stained and examined by transmission electron microscopy.

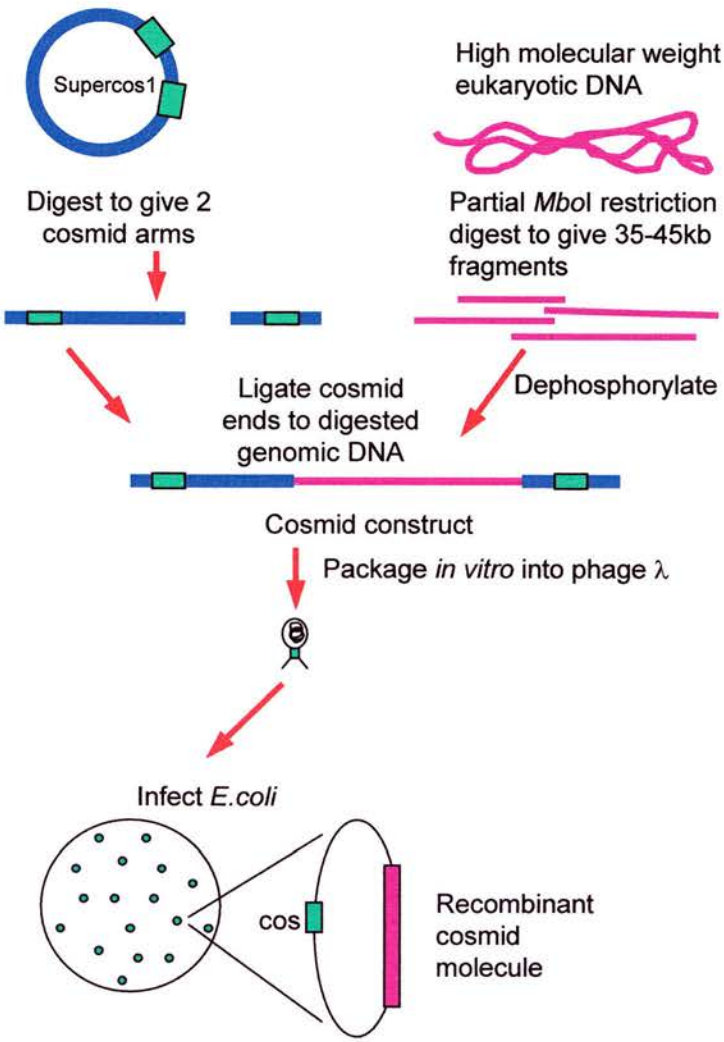
3.2 Generation of OvHV-2 Genome Sequence

3.2.1 Construction of a Cosmid Library

The study of OvHV-2 has been limited by lack of a tissue culture system supporting replication of OvHV-2. At the beginning of this work therefore, only a small amount of the genome sequence of OvHV-2 was known. A phage λ library had previously been constructed using genomic DNA from OvHV-2 infected LCL and from this a DNA clone corresponding to ORF 75 had been derived by homology to AlHV-1 (Bridgen & Reid, 1991). Recently the sequence of OvHV-2 ORF48, 49 and 73 has been derived by screening an expression library constructed using cDNA from OvHV-2 infected bovine cell lines with sheep sera (Coulter & Reid, 2002). It had been assumed that the full viral genome is present in OvHV-2 infected cell lines due to the fact that the cell lines transmit disease to cattle, sheep and deer (Reid *et al.*, 1983).

The aim of this work was to derive the complete genome sequence of OvHV-2. In the absence of a tissue culture system for production of virus, in order to derive the complete DNA sequence of OvHV-2 we were limited to using total cellular DNA from OvHV-2 infected cells instead of purified viral DNA. A cosmid library was constructed using DNA extracted from OvHV-2 infected cattle cell lines (Figure 3.2.1.1). The aim of the cosmid cloning was to produce a series of overlapping clones spanning the OvHV-2 genome. The library was predicted to contain a mixture of recombinant cosmids, some containing inserted OvHV-2 viral DNA and some containing inserted bovine LGL DNA. The aim was to identify virus containing clones by screening the cosmid library with a probe of known OvHV-2 viral sequence. The cosmid SuperCos 1 was 7.9kb in size. Since recombinant molecules of 38 - 52kb can be packaged into phage used in cosmid cloning, foreign DNA of size 30 - 44kb can be cloned using this vector. From sequence at each end of the cosmid insert, probes were generated and used to identify additional clones containing viral sequence. Since Gardella gel analysis showed that the genome is present in bovine OvHV-2 infected cell lines predominantly in the episomal or circular form (section 3.1.2), it was expected that cosmid clones spanning the viral terminal repeats might be obtained from the library.

Figure 3.2.1.1 Cosmid Cloning



The diagram shows the strategy that was used in cosmid cloning. Genomic DNA to be cloned was digested into fragments of average size 40kb by partial restriction digestion using *Mbo*I. These DNA fragments were then dephosphorylated and ligated to compatible cosmid arms. The ligated cosmid construct was packaged *in vitro* into bacteriophage heads. The recombinant bacteriophage were then used to infect *E.coli*. Once in *E.coli*, the recombinant cosmid molecule circularises and replicates like a large plasmid. The *E.coli* form colonies which can be picked and recombinant cosmid DNA isolated.

Assuming an average size of cosmid insert of 40kb, the number of transformants necessary to have a reasonable chance of identifying any given clone of interest can be calculated, using the formula

$$N = \ln(1-P)/\ln(1-f)$$

Where P is the desired probability of identifying any given clone, f is the fractional proportion of the genome in a single recombinant and N is the necessary number of recombinants. Therefore assuming the bovine genome to contain 3×10^9 bp DNA, in order to have a 99% probability of identifying a clone of interest in the library, the number of clones required would be

$$N = \ln(1-0.99)/\ln(1-(40000/3 \times 10^9)$$
$$N = 345,459 \text{ clones.}$$

However since it has been estimated that the average viral copy number per cell is 50, each viral sequence will be present in a 25 fold excess compared to unique cellular sequences, assuming 2 copies of each sequence, therefore 1/25 of this number of clones will be required. A library containing 13,818 clones would therefore be theoretically sufficient.

3.2.1.1 Preparation of Eukaryotic DNA For Cosmid Cloning

The aim was to produce eukaryotic DNA fragments of average size 40kb, as this was the correct size for ligation to cosmid DNA and *in vitro* packaging. The cosmid vector SuperCos 1MW used to clone the OvHV-2 genome contained two cos sites. These vectors are an improvement on cosmid vectors containing just one cos site, as the partially digested genomic DNA used does not need to be size fractionated (Reilly & Silva, 1993b). This means that reduced quantities of genomic DNA can be used, as DNA loss in the size fractionation step which is required using vectors with just one cos site is avoided. High molecular weight DNA was extracted from an OvHV-2 infected bovine cell line (BJ1035, cultured at the Moredun Research Institute) as described (Section 2.2.1). The extraction of high molecular weight DNA

of good quality is one of the most important steps in the construction of a cosmid library; therefore care was taken to handle the DNA solutions gently to avoid shearing the DNA. The DNA solution was mixed gently during phenol : chloroform extraction, rather than vortexing and the solution was pipetted using wide bore pipette tips.

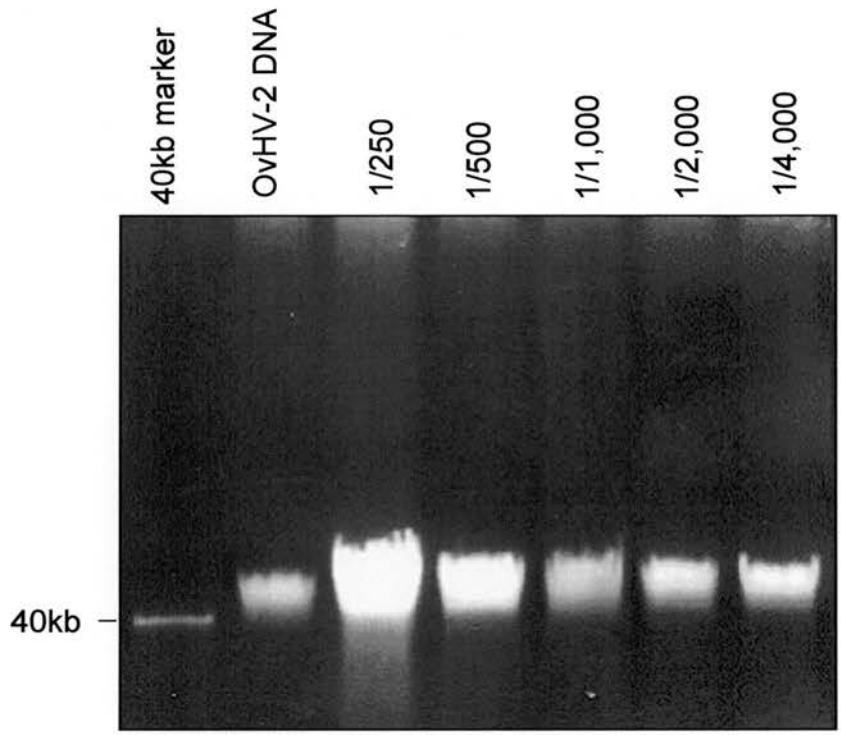
A number of dilutions of *Mbo*I (10U/μl) from 1/250 to 1/4000 were made. A 1μl aliquot of each dilution was used to digest a 10μg sample of OvHV-2 DNA to give a series of partial digests (Figure 3.2.1.2). An aliquot of the *Mbo*I digested DNA was then analysed by gel electrophoresis alongside a 40kb standard (*Sal*I linearised Cosmid A8). The restriction enzyme *Mbo*I recognises a 4bp sequence and generates a cohesive terminus, which can be ligated to DNA digested with *Bam*HI. DNA digested to a suitable size of product was then dephosphorylated in preparation for cloning into cosmids.

3.2.1.2 Generation of a Cosmid Library Using OvHV-2 DNA

In order to prepare cosmid DNA for cloning, cosmid DNA was linearised by restriction enzyme digestion with *Xba*I and dephosphorylated using CIAP to prevent self-ligation of the cosmid. The cosmid DNA was then digested with *Bam*HI to produce 2 cosmid arms (Figure 3.2.1.3). Partially digested dephosphorylated OvHV-2 DNA was ligated to cosmid arms produced as described. The product of the ligation reaction was expected to be a population of concatemers comprising OvHV-2 DNA ligated to two different cosmid arms. The ligation reaction was packaged into recombinant λ phage and the products of the packaging reaction were then used to infect the XL1-Blue MR strain of *E. coli*. SuperCos 1MW contains an ampicillin resistance gene; this was therefore used for selection of cosmid containing clones.

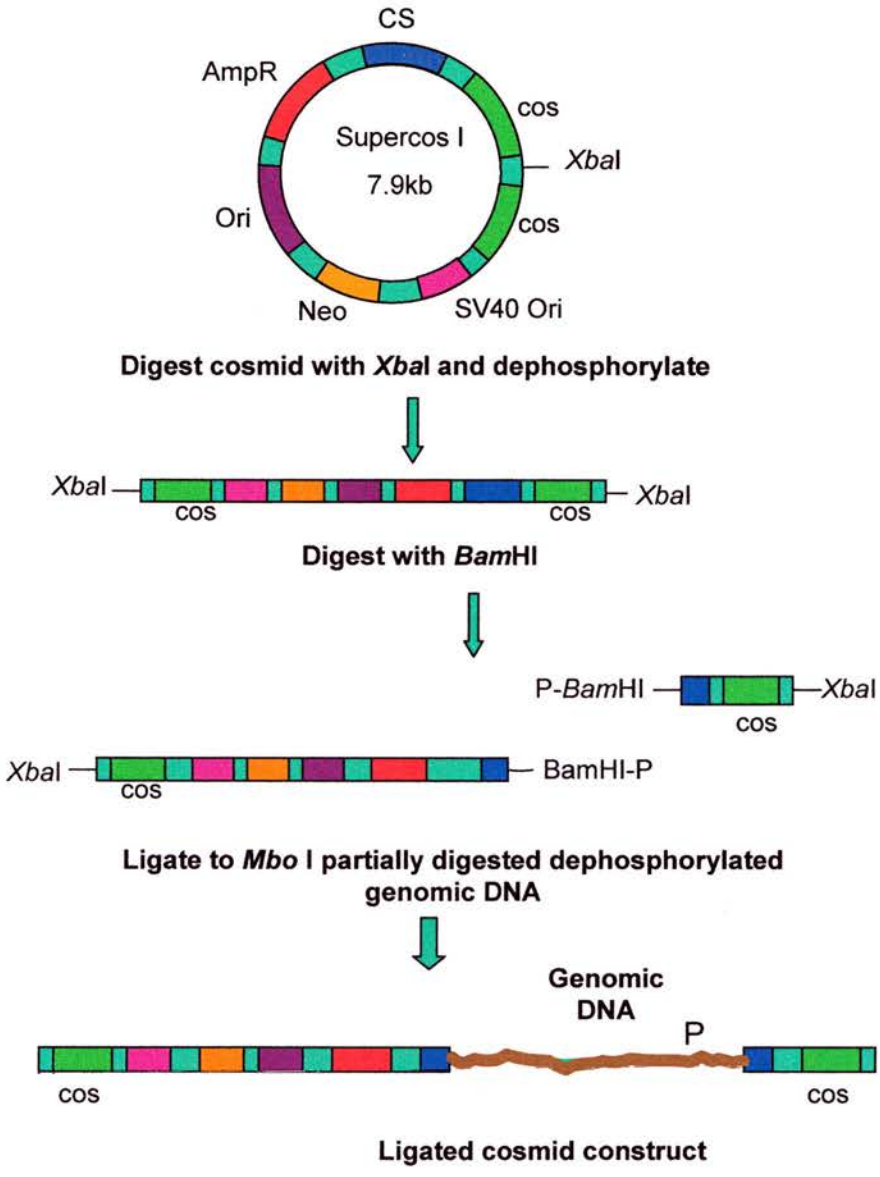
Before packaging ligated OvHV-2 DNA, the packaging efficiency was tested using the wild type λ control phage DNA. A number of trials were necessary to obtain acceptable efficiency of 400 plaques in the 10^{-4} dilution. Having established that the packaging protocol was working with acceptable efficiency using the control λ DNA, packaging was attempted using ligated OvHV-2 DNA. A number of ligations of OvHV-2 and cosmid DNA were packaged and plated in order to obtain a

Figure 3.2.1.2 *Mbo*I Partial Digests of DNA Extracted From an OvHV-2 Infected Cattle Cell Line



High molecular weight DNA was extracted from the OvHV-2 infected cattle LCL BJ/1035. Partial restriction digests of the DNA were then performed using dilutions of *Mbo*I (10U/µl) from 1/250 to 1/4,000 as labelled. A sample of each digest was analysed by electrophoresis on a 0.5% agarose gel alongside a 40kb size marker and undigested OvHV-2 DNA.

Figure 3.2.1.3 Digestion and Ligation of Cosmid DNA



Digestion and ligation of cosmid DNA in preparation for packaging into phage λ. Cosmid DNA was digested with *Xba*I and dephosphorylated, producing linearised cosmid vector. This was then digested with *Bam*HI to produce 2 cosmid arms. The cosmid arms were then ligated to dephosphorylated *Mbo*I digested genomic DNA.

library with an acceptable titre for screening. A 2μl sample of OvHV-2 DNA digested with a 1/250 dilution of M*bo*I and ligated to SuperCos 1MW was packaged. The packaging reaction diluted 1/10 and then plated resulted in 80 colonies, whilst a dilution of 1/50 resulted in 16 colonies. The sample was therefore titred at 32,000 cfu/ml. Since the library was contained in a volume of 500μl, this meant that it contained 15,000 transformants. This library was amplified and plated onto a 150mm LB amp agar plate, such that it contained many small individual colonies 0.5-1mm diameter (approximately 4,000 colonies).

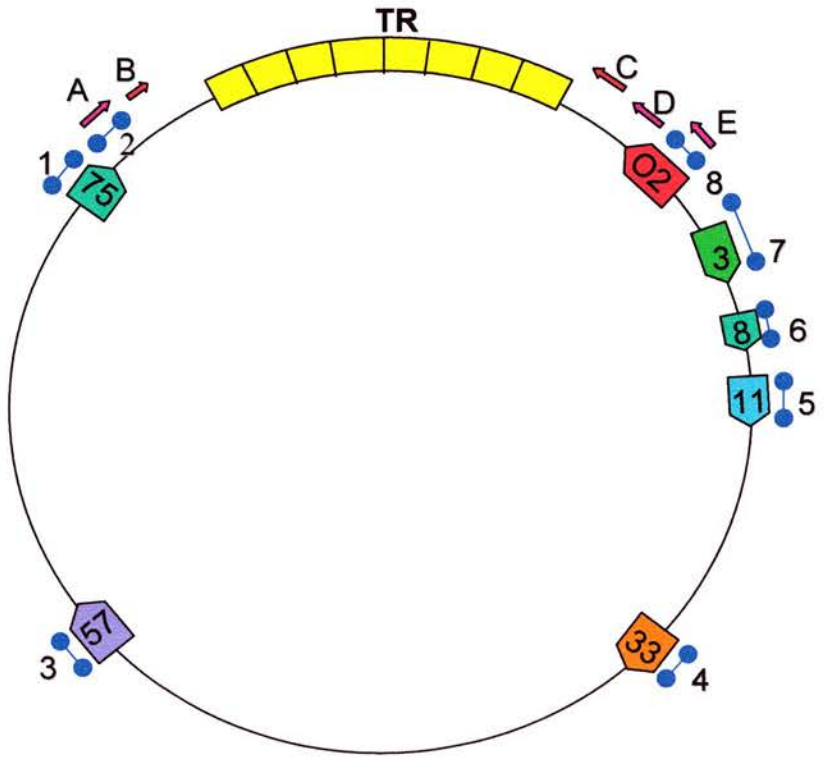
3.2.1.3 Cosmid Library Screening Using an OvHV-2 ORF 75 Specific Probe

In order to identify cosmid clones containing OvHV-2 DNA, the library was screened by colony hybridisation as described (Section 2.5.11). All probes used to screen the cosmid library are shown in figure 3.2.1.4. The probe used initially was generated by PCR amplification of a 540bp area of OvHV-2 ORF 75 using the primers O75-3' and O75-5,' and genomic DNA from OvHV-2 infected cells as a template. The PCR product was purified to remove primer and labelled with ^{32}P (Section 2.2.4). This initial screen resulted in 8 positive clones (named C75-1 to 8). These clones were colony purified by re-plating to single well-spaced colonies and re-screening. Individual positive colonies were identified, which aligned exactly with spots on the x-ray film at re-screening. These colonies were selected and cosmid DNA purified by "mini prepping". Cosmid DNA purified from these colonies was analysed by restriction enzyme digestion with *Pac*I, as this site flanked the insert at each side. Inserted DNA was found in 5 clones, C75-1.1.1, 6.1, 7.2, 1.1.2, and 7.1. Restriction digests from clones C75-1.1.1, 6.1, 7.1 and 7.2 produced a band of excised cosmid DNA at 7.9kb and bands of approximately 11kb, 25kb and 2kb. The total size of the insert was estimated to be approximately 40kb (Figure 3.2.1.5). Digestion of clone 1.1.2 resulted in a single band of approximately 12kb.

3.2.1.4 Sequencing of Positive Cosmid Clones

Modifications made to the vector SuperCos 1 to make SuperCos 1MW meant that the T7 and T3 RNA polymerase promoters had been removed (Section 2.5.1). This

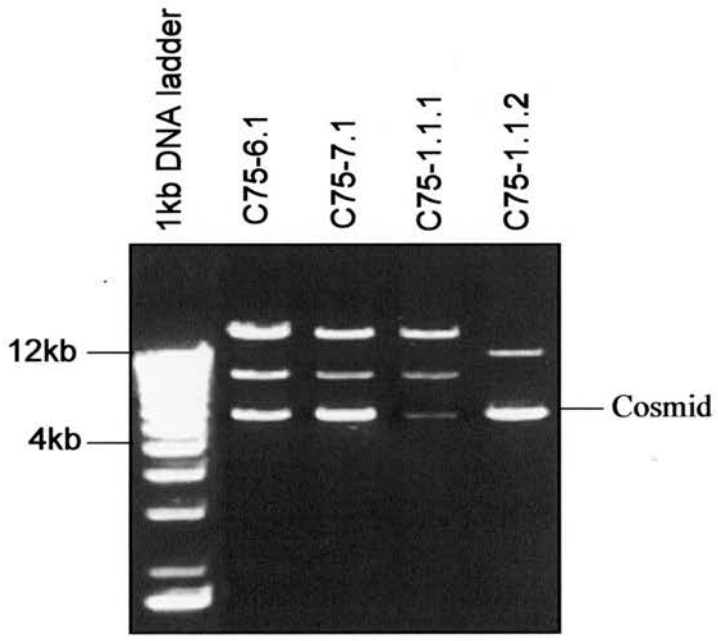
Figure 3.2.1.4 PCR Primers and Library Probes Used to Identify OvHV-2 Viral Sequence



1 – ORF 75, 2 – C75R, 3 – ORF57, 4 – ORF33, 5 – ORF11, 6 – ORF8, 7 – C249, 8 – ORF O2 A – C75R.GSP B – C75R2.GSP, C – Spl3.GSP, D – Spl2.GSP, E – O2.GSP

The OvHV-2 genome is represented circularised at the terminal repeats (TR). The position of some open reading frames are shown in coloured block arrows. The library screening probes are shown in blue and numbered, whilst the PCR primers are shown as block arrows and labelled with letters. The library probes and PCR primers were, when possible, named according to the OvHV-2 ORF sequence from which they were derived. Probe C75R and C75R.GSP were derived from sequence at the right hand end of cosmid C75. Probe C249 was derived from the AlHV-1 clone C249 (Figure 3.2.1.9) which extended from AlHV-1 ORF A3 to ORF 6. Spl2.GSP was from sequence derived by splinkerette PCR using O2.GSP, and spl3.GSP was from sequence derived by splinkerette PCR using spl2.GSP. C75R2.GSP was from sequence derived by splinkerette PCR using C75R.GSP.

Figure 3.2.1.5 *Pac*I Digests of C75 Cosmid DNA



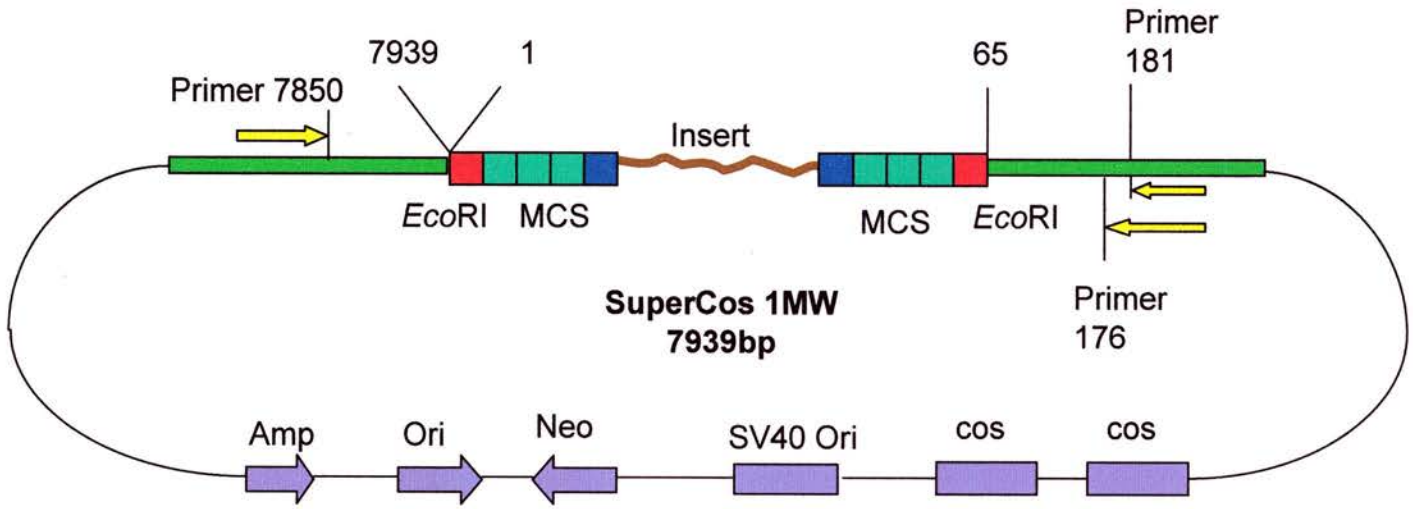
A cosmid library was constructed using OvHV-2 infected bovine cell DNA. The library was screened with an OvHV-2 ORF75 probe. Positive areas were selected and re-plated so that individual positive colonies could be picked. Cosmid DNA was prepared from positive colonies using a QIAGEN mini prep kit. The cosmid DNA was digested with *Pac*I and analysed by electrophoresis on a 0.8% agarose gel alongside a 1kb DNA ladder. Digested DNA from colonies identified by re-screening of positive areas C75-1, 6 & 7 is shown. The numbers given to each cosmid DNA sample represent the colony number given at each screen. The position of excised cosmid DNA is indicated.

meant that sequencing primers designed specifically against the T7 or T3 bacteriophage promoter regions could not be used for sequencing cosmid inserts, therefore new sequencing primers were designed and 5' end-labelled with the dye IRD800. These were named primer 7850 and primer 181 due to the coordinates on the cosmid vector at which they originated (Figure 3.2.1.6). DNA prepared from these colonies using a QIAGEN miniprep kit was sequenced. Sequence was then analysed using the program blastx, which compares the translated query sequence with the protein database nr. Sequencing using the cosmid sequencing primers 7850 and 181 generally resulted in 400-500bp of sequence from each end of the cosmid insert. Analysis of the sequence obtained from C75-1.1.1 and C75-7.1 using the sequencing primer 7850 showed the cosmids contained viral DNA homologous to AlHV-1 ORF 57.

Sequencing clones C75-1.1.1 and 7.1 using the sequencing primer 181 initially produced insufficient sequence for analysis. This could have been due to the fact that the cosmid DNA samples for sequencing were of very low concentration (usually approximately 100ng/μl), or because the sequencing primer used in the reactions was not working very well, resulting in sub-optimal sequencing reactions. The cosmid clones grew slowly in overnight culture and overnight mini cultures for preparation of cosmid DNA always resulted in cosmid DNA samples of low concentration. This may have been because the cosmids are replicated at a low copy number compared to smaller plasmids. In addition because they are large molecules, cosmids have a relatively long replication time. The long replication time would result in slower growth of the host bacteria. It is also possible that the viral DNA was toxic to the bacterial hosts, resulting in slower bacterial growth.

In order to make a more concentrated cosmid DNA preparation, cosmid DNA was purified by making a large cosmid DNA preparation using a QIAGEN large construct kit as described (Section 2.1.18). The left hand end of this DNA was sequenced using primer 181 and this time sufficient sequence was obtained for analysis. Comparison of this sequence with the database revealed no similarity to database protein sequences, but due to the fact that the probe used to identify this cosmid was ORF 75, it was assumed to be in the area beyond ORF 75, possibly between open reading frames. This meant that cosmid C75-1.1.1 was approximately

Figure 3.2.1.6 Location of the Sequencing Primers for SuperCos 1 MW



The map of Supercos 1 MW is shown with the region around the multiple cloning site (MCS) enlarged. The sequencing primers are shown as yellow arrows, with the direction of the arrow indicating the direction of the sequencing reaction using the primer. The coordinates of the multiple cloning site and the 3' end of the sequencing primers are shown. The OvHV-2 DNA insert to be sequenced is shown in brown.

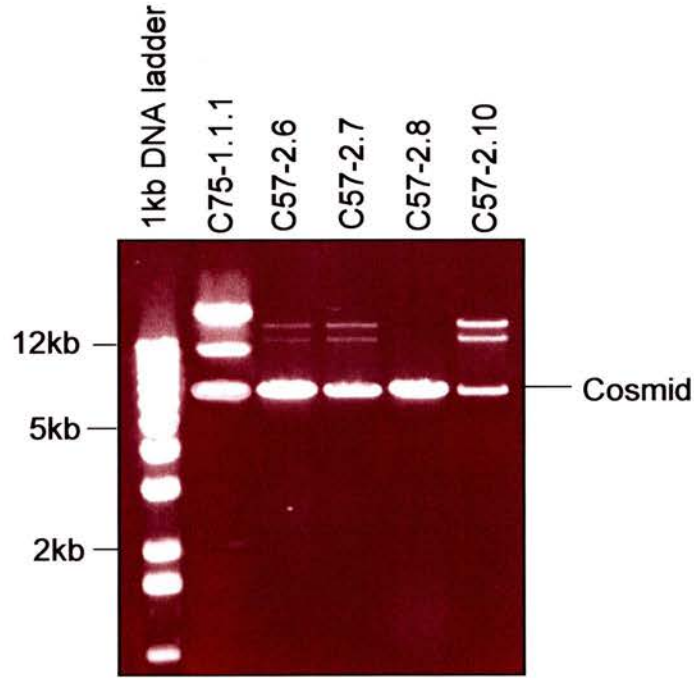
40kb in size and spanned 17 ORFs. Sub-optimal sequencing reactions when using primer 181 might have been due to inefficient binding of the sequencing primer to the target sequence. It was suspected that this could have been because the sequencing primer was too short. In order to rectify this problem, a new longer sequencing primer was designed from the SuperCos 1MW sequence and was named primer 175 (Figure 3.2.1.6). This replaced primer 181 in all subsequent sequencing reactions, however sequence obtained from this primer continued to be weak and difficult to read compared with sequence obtained using primer 7850. It was concluded that there may be secondary structure in this region of the cosmid which made primer binding difficult.

Increasing the concentration of the DNA preparation used for sequencing usually improved the sequence yield. The protocol of isolation of cosmid DNA using the QIAGEN large construct kit was time consuming and the yields of DNA were disappointing. It was found that cosmid DNA concentrated using Microcon centrifugal filter units (Section 2.1.13) resulted in cosmid DNA of sufficient concentration for sequencing, therefore this method was used to concentrate all cosmid samples before sequencing.

3.2.1.5 Screening The Cosmid Library Using OvHV-2 ORF 57

In order to extend the viral genome sequence to the left of the known virus sequence from clone C75-1.1.1, a new "walking" probe was made by PCR amplification of the ORF 57 sequence at the left hand end of clone C75-1.1.1 in order to re-screen the library. PCR primers were designed using the ORF 57 sequence at the end of cosmid C75-1.1.1 to amplify a sequence spanning 50bp to 533bp from the left hand end of cosmid C75-1.1.1, resulting in a product of 483bp (Appendix 2). The probe was made by PCR using C75-1.1.1 DNA as a template. This probe was used to screen the cosmid library and 1 large plate with 4,000 colonies was probed as before. From this first screen, 10 positives were re-plated and re-screened. Cosmid DNA purified from these was analysed by restriction digestion using *Pac*I. All positive colonies digested to the same 2 products of approximate size 20kb and 30kb (Figure 3.2.1.7) in addition to the 7.9 kb product of cosmid DNA. Digestion of clones such as C57-2.6 and 2.7 with *Pac*I resulted in a cosmid band of disproportionally high intensity

Figure 3.2.1.7 Digestion of C57 Cosmid Clones Using *Pac*I



The cosmid library was probed using an OvHV-2 ORF 57 probe. Positive areas were re-plated so that single positive colonies could be selected. Cosmid DNA was prepared from individual positive colonies using a QIAGEN mini prep kit. The cosmid DNA was digested using *Pac*I and the products analysed by electrophoresis on a 0.8% agarose gel alongside a 1kb DNA ladder and C75-1.1.1 DNA. The cosmid DNA shown resulted from re-plating of positive area C57-2. The numbers given to each cosmid DNA sample represent the colony number at each screen. The position of excised cosmid DNA is indicated.

compared to the other bands produced on digestion. This suggested that the clones used to isolate the cosmid DNA might have contained two different types of cosmid sequence. This could have resulted from selecting more than one clone when picking colonies, however this was unlikely as the colonies selected were well spaced on the culture plate. It is possible that during growth in culture the cosmid DNA in some clones was rearranged and the insert lost. This would result in a mixed culture of bacteria, with some clones containing cosmid DNA without insert. These bacteria were likely to have a selective advantage over the bacteria containing cosmids with inserted DNA, resulting in quicker growth of these bacteria. This therefore was evidence that some cosmid containing clones were unstable and it was hypothesised that instability of cosmid inserts may be a cause of failure to isolate some viral sequences if this problem were to occur.

The ends of a number of these clones were sequenced. Sequence obtained from clone C57-2.10 using cosmid sequencing primer 175 showed homology with AlHV-1 ORF 33. Sequence obtained using primer 7850 was homologous to AlHV-1 ORF63. This meant that by analogy with the ORF layout of AlHV-1, the cosmid spanned an area of 33 open reading frames and had a size of approximately 40kb. The overlap with cosmid C75-1.1.1 was seven ORFs and approximately 10kb, therefore the screen resulted in a cosmid containing 30kb of new sequence.

3.2.1.6 Screening The Cosmid Library Using a C75R Probe

In order to extend the virus sequence towards the viral terminal repeats to the right of ORF 75, the library was re-screened using a probe designed from sequence at the right hand end of cosmid C75. PCR primers were designed from sequence of the right hand end of clone C75-1.1.1 and were named C75R-5' and C75R-3'. These primers amplified a 435bp region spanning 25bp to 461bp from the right hand end of cosmid C75-1.1.1 (Appendix 2). A probe was made by PCR amplification using C75-1.1.1 cosmid DNA as a template. This probe was named C75R and was used to screen one plate of 4,000 colonies. Four positive spots were re-plated and screened, initially analysis of sequence obtained using primer 7850 revealed weak homology with AlHV-1 ORF 33. It was therefore envisaged that this clone might span the terminal repeats and this cosmid was named cosmid C75R-1. Some of the C75R-1

sequence was ambiguous, therefore area was re-sequenced and the ambiguous areas checked. Re-analysis revealed no significant homology with viral sequences.

Despite analysis of the sequence at the right hand end of cosmid C75R revealing no homology to virus sequence, it was still suspected that this clone might span the viral terminal repeats and therefore might contain the potential OvHV-2 virulence genes at the left hand end of the virus genome. In order to further characterise the clone, it was decided to analyse C75R-1 by restriction mapping. The first step undertaken was to try to identify the sequence overlapping with the left hand end of clone C75-1.1.1.

In order to analyse cosmid C75R-1 by restriction mapping, cosmid DNA from the clone was restriction enzyme digested using a series of enzyme digests; *Sal*I/*Bam*HI, *Bam*HI, *Not*I/*Bam*HI, *Hin*dIII, *Not*I/*Hin*dIII, *Eco*RI, *Not*I/*Eco*RI, *Sal*I, *Not*I/*Sal*I, *Not*I. The products of the digests were electrophoresed on a 1% agarose gel and analysed by Southern blotting for hybridisation with the hybridisation probe C75R (from sequence at the right hand end of the genome beyond ORF 75), in order to establish a section of cosmid overlapping with the end of cosmid C75-1.1.1 from where the probe originated.

On the first hybridisation the probe annealed to many bands on the gel, including the bands of SuperCos 1MW DNA excised from the cosmid clones, which should not have cross hybridised with the C75R probe. It was suspected that this might have been caused by contamination of the probe with template, which in this case was cosmid C75-1.1.1. The probe was re-synthesised by PCR amplification, using OvHV-2 DNA as a template and the blot re-probed. On this probing no hybridisation was detected, therefore there seemed to be no areas of overlap with C75-1.1.1. This confirmed the suspicion from re-sequencing that the clone was not of viral origin, therefore likely to be a clone of bovine cellular DNA.

Southern analysis using the probe C75R made by PCR amplification using cosmid as a template PCR resulted in hybridisation to all DNA containing cosmid sequence. It was thought that screening of the library using this probe might have resulted in the false positive clone C75R-1 in the same way. This would be due to contamination of the PCR product with template, in this case cosmid C75-1.1.1 and labelling of this cosmid in the labelling reaction. Labelled cosmid would cross-

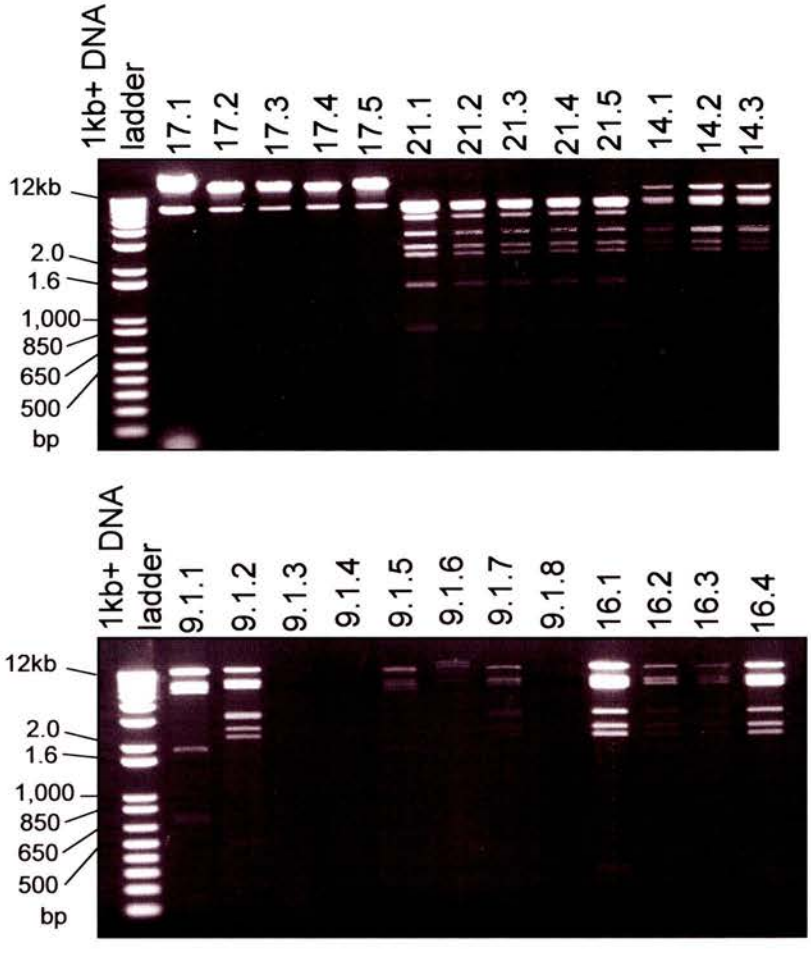
hybridise with any cosmid clones, whatever the insert. To avoid this problem in future, it was decided that all probes used should be produced by PCR amplification of OvHV-2 genomic DNA rather than cosmid and the products gel purified in order to remove PCR product from template. The probe C75R was re-made by PCR amplification using DNA from OvHV-2 infected cell lines as a template. The resulting PCR product was electrophoresed on an agarose gel and the PCR product purified from the gel (Section 2.1.6). This was carried out to ensure that the DNA used as a probe was not contaminated with the template used for PCR. The gel purified C75R probe was labelled with P^{32} and was used to screen the library. All resulting positive clones had the same restriction profile as C75-1.1.1 when digested with *Pac*I. This meant that the screen did not identify a new cosmid clone.

3.2.1.7 Screening the Cosmid Library Using an OvHV-2 ORF 33 Probe

In order to extend sequence at the right hand end of the genome, PCR primers were designed against the ORF 33 sequence. The primers were from 13bp to 398bp from the left hand end of cosmid C57 and amplified a product 385bp in length (Appendix 2). The probe was made by PCR amplification using OvHV-2 DNA as a template. The ORF 33 PCR proved difficult to optimise. The PCR was optimised such that a single band was produced, however this was of low intensity and purification of the product resulted in a probe of low DNA concentration (8ng/μl). This probe was used to probe the library, (4 large plates, 16,000 colonies) and no positives found on this screen.

The PCR was further optimised such that a band of the correct size with more product was produced, however this also resulted in the production of spurious bands, therefore the whole PCR product was electrophoresed and band of the correct size was excised from the gel. The DNA was then purified from the gel as described (Section 2.1.6) and labelled with ^{32}P. The new probe was then used to re-probe the library and a further 9 large plates screened (36,000 colonies). From this screen, 15 positives were re-plated and re-screened and 25 colonies from this screen were selected. Cosmid DNA was purified from these clones and analysed by restriction enzyme digestion with *Pac*I and with *Eco*RI. The products of the digests were analysed by agarose gel electrophoresis and 4 new clones identified (Figure 3.2.1.8).

Figure 3.2.1.8 Restriction Digests of C33 Cosmid DNA With *Eco*RI



The cosmid library was probed using an OvHV-2 ORF33 probe. Positive colonies (numbered C33.9, 14, 16, 17 & 21) were picked and re-plated so that single colonies could be identified. Some colonies were re-plated a third time. Cosmid DNA was prepared from single bacterial colonies using a QIAGEN mini prep kit. The cosmid DNA was digested with *Eco*RI and the products analysed by electrophoresis on a 0.8% agarose gel alongside a 1kb+ DNA ladder. The numbers given to each cosmid DNA sample represent the colony number on each screen.

These clones were named C33-9, 14, 16, 17 and 21 according to the colony number given at the first library screen. Digestion of cosmid DNA extracted from the clones C33-9, 14 and 21 with *Eco*RI resulted in the same restriction pattern, suggesting the sequence of these clones was the same. The cosmid ends of clones C33-16, 17 and 21 were sequenced and two clones contained viral DNA with homology to the AlHV-1 genome. The sequence obtained from C33-17 was not similar to any known viral sequence therefore the clone was assumed to contain non-viral DNA. The sequence obtained from clone C33-21 using primer 7850 was homologous to AlHV-1 ORF22, however there was insufficient sequence from the reaction with primer 175 for analysis. The sequence from this primer was likely to be in the region to the right of ORF 33, towards ORF 57. This cosmid therefore contained approximately 15kb of new sequence, spanning 10 new OvHV-2 open reading frames. The sequence obtained from C33-16 using primer 7850 was homologous to AlHV-1 ORF 11, whilst the sequence obtained using primer 175 was homologous to AlHV-1 ORF40. This meant that by analogy with the ORF layout of AlHV-1, the cosmid was approximately 40kb in length and spanned 27 open reading frames. The overlap of the new C33 cosmid sequence with cosmid C57 was 12 open reading frames and approximately 10kb; therefore the screen generated 30kb of new OvHV-2 sequence.

3.2.1.8 Screening the Cosmid Library Using OvHV-2 ORF 11

In order to extend sequence further at the right hand end, a probe was made from the sequence of OvHV-2 ORF 11. The primers O11-5' and O11-3' (Appendix 2) were designed to amplify a product extending from 67bp to 374bp from the left hand end of cosmid C33. The probe was made by PCR amplification using OvHV-2 DNA as a template. The PCR product was electrophoresed and the required band excised from the gel. The PCR product was then purified from the gel. This probe was used to screen the library; 6 large plates containing approximately 24,000 colonies were screened. From this screen, 9 positives were re-plated and 17 positives selected on the second screen. From the 5 samples sequenced, C11-9 and C11-12 both contained viral DNA of the same clone. The sequence obtained using primer 175 had homology to AlHV-1 ORF 10 and the sequence obtained using primer 850 had homology with ORF42. Assuming the layout of the genome to be similar to that of

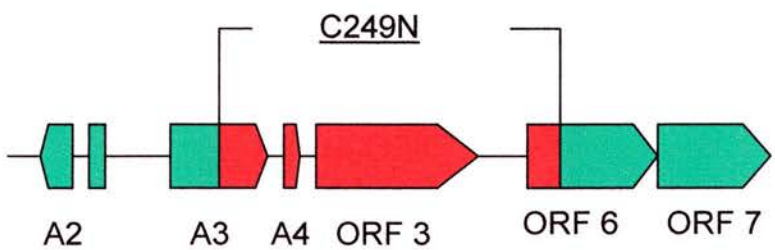
AlHV-1, the screen had only advanced 1 open reading frame. This was a distance of approximately 1kb.

3.2.1.9 Screening The Cosmid Library With The AlHV-1 Clone C249

The aim was to extend sequence further into the left hand end of the genome and if possible over the terminal repeats, however probing with ORF 11 had resulted in further sequence of just one ORF distance. A probe whose sequence was located closer to the terminal repeats was therefore needed. Analysis of cosmid sequence so far revealed significant amino acid homology of much of the OvHV-2 sequence with that of AlHV-1, therefore it was hypothesised that there may be sufficient homology at the nucleotide level to screen the library using a probe containing AlHV-1 sequence. A clone containing sequence from the left hand end of AlHV-1 was obtained (C249N). This extended from ORF A3 to ORF 3 and was 7kb in size (Figure 3.2.1.9). The clone consisted of C249N cloned into the plasmid pBluescribe M13+. The C249 fragment was excised from the vector by digestion with *Sac*I and *Sph*I. The products of the digest were then electrophoresed and the band of interest excised. The C249N DNA was then purified from the gel and labelled with ^{32}P for use as a library screening probe.

The library was plated onto 6 large plates (24,000 colonies approximately). These were probed using colony hybridisation as before, but blots were hybridised at low stringency (37°C) using Ultrahyb hybridisation buffer (Section 2.2.6). Low stringency hybridisation was used because the probe was likely to have lower homology with the OvHV-2 sequence than the screening probes used so far. This was due to the fact that the probe sequence was AlHV-1 DNA and not OvHV-2. From this screen, 21 positives were re-plated and re-screened. Screens generally resulted in a large number of possible positives of varying intensities on the x-ray film. A sample of 25 possible positives was selected and the colonies re-plated onto 2 identical plates. Since the aim was to identify a clone which extended from ORF 11 over to C75R spanning the viral terminal repeats, one plate was probed by colony hybridisation using the OvHV-2 C75R probe, and the other plate probed using the OvHV-2 ORF 11 probe. Hybridisation was performed using Ultrahyb hybridisation buffer at the normal stringency hybridisation temperature of 42°C. There were no convincing positives on this screen. Although a new clone may not have necessarily

Figure 3.2.1.9 Location of the Clone C249N on the AlHV-1 Genome



The clone C249N was isolated from molecular cloning of the AlHV-1 genome (Ensser *et al*, 1997). The diagram represents part of the genome of AlHV-1, with the open reading frames shown in solid blocks. The open reading frames in the clone C249N are shown in red. The clone was approximately 7kb in size, and extended from AlHV-1 ORF A3 to ORF6.

overlapped with either C75R or C33, it was concluded low stringency hybridisation was resulting in the identification of false positive clones.

3.2.1.10 Screening The Cosmid Library With OvHV-2 ORF8

Screening the library using the AlHV-1 C249 probe failed to result in the identification of new virus containing clones. This was probably due to insufficient similarity between the clone and the target DNA. New OvHV-2 ORF8 PCR primer sequences published by Dunowska *et al.*, (2001) made it possible to produce an OvHV-2 ORF8 screening probe, in order to try to identify sequence to the left hand end of ORF 10. The PCR primers were designed to amplify a region between –116 and +91 relative to the ORF8 sequence, producing a product of length approximately 2.8kb. This probe was used to screen 24,000 colonies on 6 large plates. From the second screen cosmid DNA from 19 colonies was analysed by restriction enzyme digestion with *Pac*I and *Eco*RI. On analysis sequence obtained from sample C8-5.2 using primer 7850 had high homology to AlHV-1 ORF6. Sequence obtained from C8-18.4 using primer 7850 had homology with AlHV-1 ORF A2. By analogy with the ORF layout of AlHV-1 the screen had produced sequence of 9 open reading frames of new OvHV-2 sequence (approximately 20kb) extending to the left hand end of ORF 10. Sequence of the right hand end of these cosmids using sequencing primer 175 did not produce enough sequence for database comparisons, however restriction enzyme analysis and gel electrophoresis showed cosmids C8-5.2 and C8-18.4 to be approximately 40kb in size.

3.2.1.11 Screening The Cosmid Library With OvHV-2 ORF O2

It was expected that screening the library with an OvHV-2 O2 probe could result in the identification of a clone that either extended into, or spanned the viral terminal repeat sequences. A PCR probe was made from the sequence of ORF O2, this was designed to amplify sequence from 3bp to 400bp from the left hand end of cosmid C8-18.4, resulting in a product of 387bp (Appendix 2). The probe was made by PCR amplification using OvHV-2 DNA as a template. The PCR product was electrophoresed and the PCR product purified from the gel as described. The library was probed using the ^{32}P labelled O2 probe and 6 large plates (24,000 colonies) were screened. The second screen resulted in 7 positive colonies and cosmid DNA was

purified from these. On restriction analysis with *Eco*RI, 6 out of the 7 cosmid DNA samples resulted in the same restriction pattern as cosmid C8-18.4 and the remaining sample contained no insert. This screen had therefore not resulted in the identification of any new virus containing cosmids.

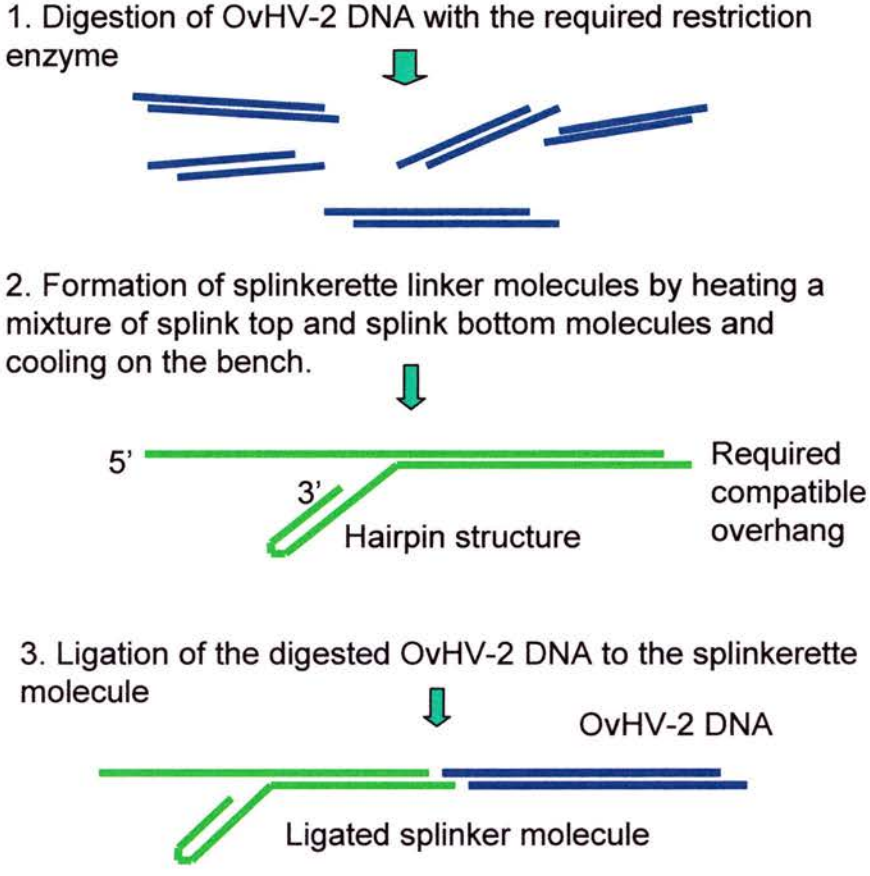
3.2.2 Extension of Known Sequence Using PCR

Screening the library with probes to extend the sequence at both the right hand end (C75R probe) and the left hand end (O2 probe), of the virus genome resulted in no new sequence. This was possibly because the cosmid library did not contain clones with inserts of viral terminal repeat DNA. It is possible that these clones do not grow well in culture and were not amplified in sufficient numbers when the cosmid library was amplified.

Although it was not possible to identify all parts of the virus genome in cosmid clones, it was still hypothesised that DNA extracted from the OvHV-2 infected bovine cell line BJ/880 contained complete circular viral genomes. It was therefore decided to use the known OvHV-2 sequence obtained from cosmid cloning, to extend the viral sequence using a PCR based walking method on BJ/1035 OvHV-2 DNA. The technique of splinkerette PCR was chosen as a suitable method. This method enables the application of PCR amplification to stretches of DNA where the sequence information is only available at one end. Splinkerette PCR is a modification of vectorette PCR; both methods have been used for PCR walking (Devon *et al.*, 1995) and in the isolation of end fragments from YAC recombinants.

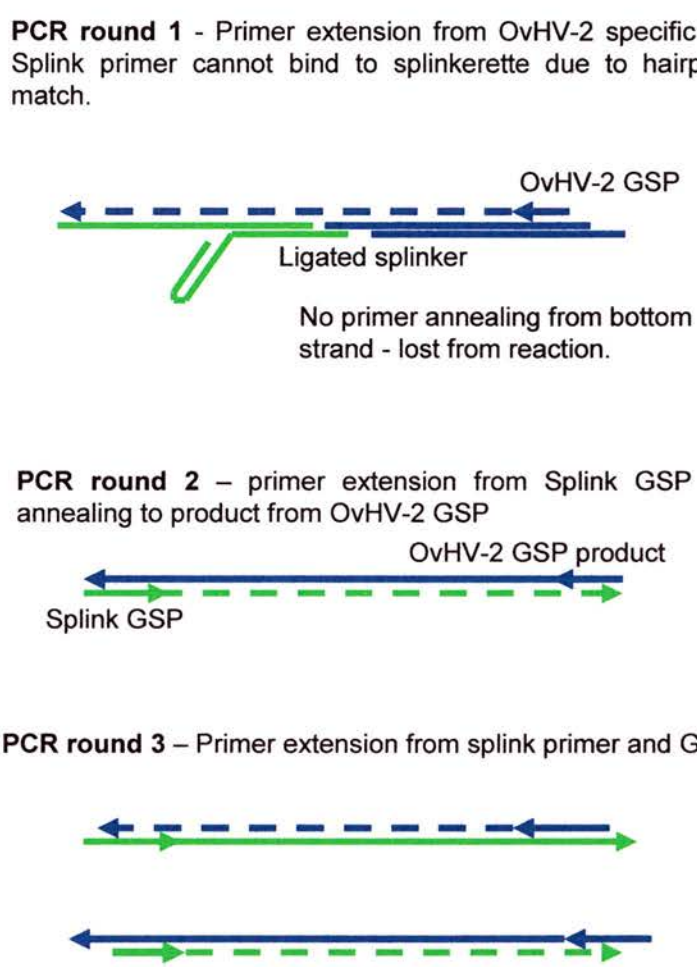
The aim was to extend the sequence to the terminal repeats at both ends of the genome in order to obtain the complete OvHV-2 viral sequence. Although splinkerette PCR does not generally result in as large walking "steps" as in cosmid cloning, if the genome layout of OvHV-2 is similar to that of AlHV-1, the distance to the viral terminal repeats was expected to be only 2 - 3kb. Splinkerette PCR involves restriction enzyme digestion of the DNA of interest and ligation of this DNA to a "splinkerette linker molecule". The steps used in the generation of ligated splinkers are shown diagrammatically in figure 3.2.2.1. PCR amplification is then performed using primers specific for the known genomic DNA sequence (gene specific primers or GSPs), and for the splinkerette sequence (Figure 3.2.2.2). The splinkerette primer

Figure 3.2.2.1 Splinkerette PCR – Formation of Ligated Splinker Molecules



Step 1: DNA (shown in blue) was extracted from OvHV-2 infected cattle cell line BJ/1035 was digested with the required restriction enzyme. **Step 2**: Splinkerette linker molecules were made by mixing equimolar amounts of top strand oligonucleotide and bottom strand oligonucleotide (shown in green), heating to 90°C and cooling at room temperature. This created a linker molecule with the required compatible overhang for ligation. The 3' end of the bottom strand of the linker molecule was designed so that it formed a hairpin structure and was not complementary to the 5' end of the top strand. **Step 3**: Splinkerette linker molecules were ligated to the digested OvHV-2 DNA using T4 DNA ligase enzyme to form ligated splinker molecules.

Figure 3.2.2.2 Splinkerette PCR



PCR amplification was performed using the ligated splinkerette molecule as template. The primers used were a gene specific primer (GSP)(blue arrow) of choice and a splinkerette GSP (splink GSP)(green arrow) which was designed to anneal to the known splinkerette sequence. **PCR round 1** – Primer extension proceeds from the OvHV-2 GSP. The splinkerette primer cannot anneal due to the mismatch in the bottom strand of the splinkerette linker. Primer extension can therefore only occur from splinkerette molecules ligated to target DNA. **PCR round 2** – the splinkerette GSP anneals to the product from round 1 and primer extension occurs. **PCR round 3** – primer extension proceeds from both primers.

is the same sequence as the top strand of the splinkerette molecule, however the bottom strand of each linker molecule forms a hairpin like structure at the 3'end. This means that the splinkerette primer cannot anneal to the splinkerette bottom strand, as this strand is mismatched. The bottom splinkerette strand is therefore removed from the reaction. PCR extension using the splinkerette primer can only occur when a bottom strand has been synthesised by chain extension from a digested genomic DNA ligated to a splinkerette linker. This design prevents non-specific PCR products occurring due to amplification of unligated splinkerette molecules. The PCR is then repeated using internal primers. Any resulting PCR product is then cloned and sequenced.

3.2.2.1 Splinkerette PCR using OvHV-2 O2 Primers

OvHV-2 DNA was digested initially with *Eco*RI, *Bam*HI, *Xba*I or *Hin*dIII. The digested DNA was then ligated to "linker molecules". Linker molecules were constructed by annealing oligonucleotides as described (Section 2.4.5). The linker molecules required a unique bottom strand, depending on the sticky end that was required. Linker molecules for *Eco*RI, *Bam*HI and *Xba*I all used the same top strand (Splink Top), whilst the top strand used for the *Bam*HI linker (Splink Top *Bam* HI) differed by one base pair from the Splink Top sequence (Appendix 4).

The ligated DNA samples were used as a template for PCR using a primer against the known linker sequence and a gene specific primer (Appendix 3). The positions of all splinkerette primers on the OvHV-2 genome are shown on figure 3.2.1.4. The primer used in the initial PCR was the gene specific primer O2. This primer was designed to amplify sequence of O2 towards the left hand end of the viral genome and was designed to the complement of the O2 sequence. The 5' end located 70 bp downstream of the *Bam*HI site at left hand end of cosmid O2, and the 5' end of the internal primer was located 47bp from the *Bam*HI site.

PCR was performed using the O2.GSP primer and the splinkerette primer (splink primer), using a combined "hotstart" and "touchdown" program (Section 2.4.7) to increase specificity of the reaction. In this reaction, the initial annealing temperature used was above the expected annealing temperature of the primers. The annealing temperature was then reduced in each cycle of PCR. The PCR was

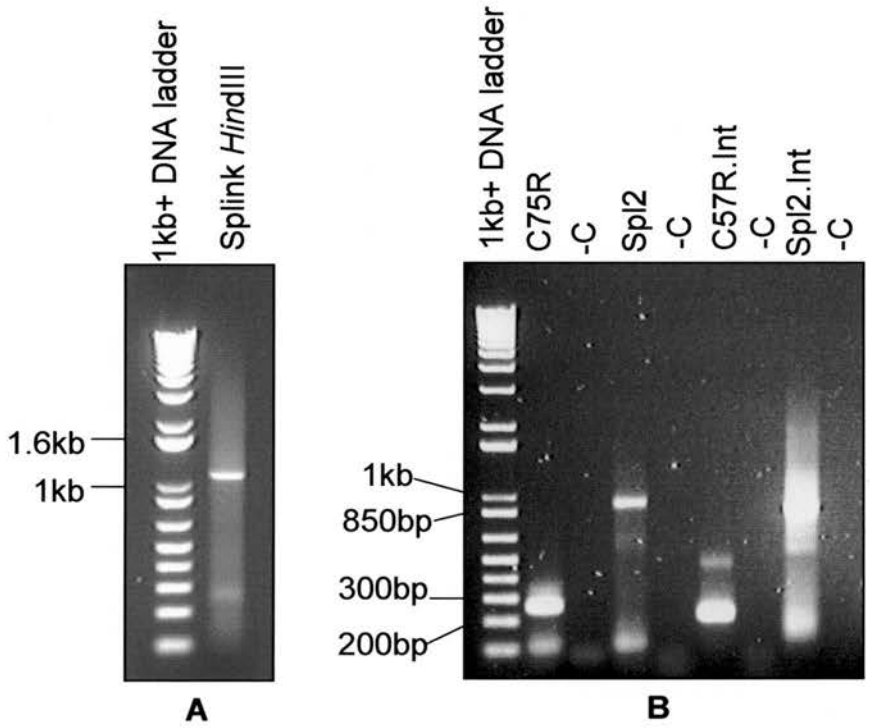
repeated using the splinkerette and O2 internal primers using the same PCR program as was used in the first round. PCR using the ligated *Hin*dIII OvHV-2 digest as a template resulted in a product of approximately 1.2kb using the internal primers (Figure 3.2.2.3). This product was cloned into a pCR 2.1-TOPO TA vector and sequenced. The sequence was analysed using the "pairwise blast" program, in which two sequences are compared with each other (Section 2.6.1). The sequence was found to overlap with known sequence of O2. The sequence was therefore concluded to be viral; therefore this round of PCR had resulted in 1.2kb of new OvHV-2 viral sequence at the left hand end of the genome beyond O2. This clone was named splink1. Comparison of the sequence with the Genbank database using the program blastx showed the sequence was composed partly of sequence with homology to AlHV-1 A2, however no sequence homologous to A1 was identified, therefore it was assumed that the left hand end of the new clone was in sequence beyond O2.

3.2.2.2 Splinkerette PCR Using Spl2 Primers

In order to extend the known virus sequence further towards the left hand end of the genome beyond the splink1 sequence, PCR primers were designed using the splink1 sequence and named spl2.GSP and spl2.GSP.Int (Appendix 3) (Figure 3.2.2.4). The spl2.GSP primer 5'end was 69bp from the ligation point of the splink1 sequence to the splinkerette linker. The 5' end of the spl2.GSP.Int primer was 23bp from the splinkerette ligation point. PCR amplification using the gene specific primer spl2.GSP and the splinkerette primer, using the *Bam*HI digested and ligated OvHV-2 DNA as a template, resulted in a 900bp product (Figure 3.2.2.3).

The PCR was repeated using the internal primers and the product was cloned and sequenced. The sequence was compared to the splink1 sequence using the pairwise blast program as before. The new sequence shared overlap with splink1 sequence against which the spl2.GSP primers were designed; therefore the sequence was assumed to be specific OvHV-2 viral sequence and was named splink2. Comparison of the splink2 sequence with the Genbank database using the program blastx did not show any homology of sequence with known viral sequence. This sequence was therefore assumed to be new OvHV-2 sequence to the left of ORF O2 in between open reading frames.

Figure 3.2.2.3 Products from Splinkerette PCR of OvHV-2 DNA



OvHV-2 DNA was digested with a number of enzymes and ligated to splinkerette linker molecules. The ligated DNA was then used as a template for PCR amplification. The products were analysed by electrophoresis on a 1% agarose gel.

A - Products of PCR amplification of ligated *Hin*dIII digested OvHV-2 DNA using the splink primer and the O2.GSP.

B - Products of PCR amplification of *Bam*HI digested and ligated OvHV-2 DNA. C75R – PCR Product using the splink primer and C75R.GSP. C75R.Int – Product from amplification of C75R with internal primers splink.Int and C75R.GSP.Int. Spl2 – PCR product using the splink primer and spl2.GSP. Spl2.Int – Product from amplification of spl2 using internal primers splink.Int and spl2.GSP.Int. –C - PCR negative control for each primer pair using distilled water as a template.

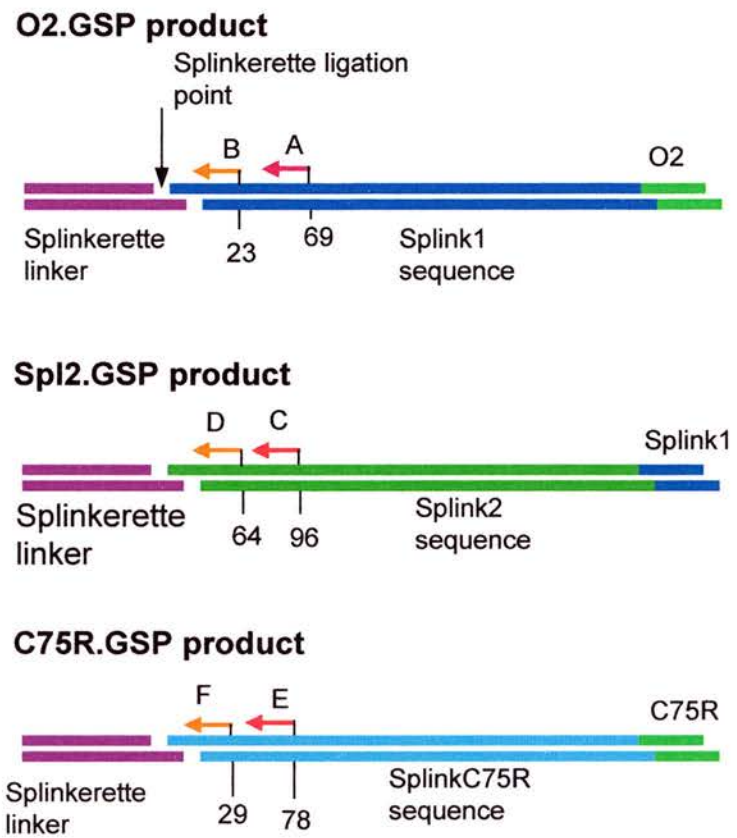
3.2.2.3 Splinkerette PCR Using C75R Primers

In order to extend the known virus genome sequence to the right hand end of the viral genome beyond the right hand end of cosmid C75-1.1.1 towards the viral terminal repeats, gene specific primers were designed against the sequence at the right hand end of cosmid C75-1.1.1 beyond ORF 75, and were named C75R.GSP and C75R.GSP.Int. The 5' end of primer C75R.GSP was located 45bp downstream of the *Bam*HI site at the right hand end of cosmid C75-1.1.1, and the 5' end of the C75R.GSP.Int primer was 32bp downstream of the *Bam*HI site. PCR amplification using the C75R.GSP primer and the splinkerette primer, using the BamHI digested and ligated OvHV-2 DNA as a template resulted in a 200bp product (Figure 3.2.2.3). The PCR was repeated using the internal primers and the product cloned and sequenced. The sequence was compared with the C75R sequence and was found to overlap with the right hand end of the C75R sequence. Comparison of the sequence with that of the Genbank database did not reveal any homology with known viral sequence. The sequence was therefore assumed to be new OvHV-2 sequence between open reading frames beyond the C75R sequence and was named splinkC75R. The genome of AlHV-1 contains an area beyond ORF 75, between A9 and A10, which does not contain any identified open reading frames. It was assumed that the C75R sequence and the splinkC75R sequence were located in this region.

3.2.2.4 Splinkerette PCR Using Spl3 Primers

Gene specific primers were designed against the splink2 sequence and named spl3.GSP and spl3.GSP.Int (Appendix 3) (Figure 3.2.2.4). The 5' end of the spl3.GSP primer was located 96bp from the ligation point to the splinkerette linker, and the 5' end of the spl3.GSP.Int primer was located 64bp from the splinkerette ligation point. The primers were designed to extend the viral sequence towards the terminal repeats beyond the spl2 sequence. PCR using the spl3.GSP primer and the splinkerette primer and each of the 4 ligated OvHV-2 digests (*Hin*dIII, *Bam*HI, *Xba*I, *Eco*RI) as templates, did not result in any product either on the first round of PCR, or on the second round using internal primers.

Figure 3.2.2.4 Design of Primers for Splinkerette PCR



GSP – gene specific primer, **Int** – internal primer
A – Spl2.GSP **B** – Spl2.GSP.Int **C** – Spl3.GSP
D – Spl3.GSP.Int **E** – C75R2.GSP **F** – C75R2.GSP.Int

The splinkerette PCR products are shown, and the GSP used to produce each product is indicated above each diagram. The splinkerette linker sequence (purple) is shown at the left hand end of all products. The position of the splinkerette ligation point is shown and the coordinates of primers are shown as distances in base pairs of the 5' end of each primer from the splinkerette ligation point. The spl2 primers were designed from sequence obtained by splinkerette PCR using the O2.GSP. The new splink1 sequence is shown in blue, with O2 sequence (green) to the right of the splink1 sequence. The spl3 primers were designed from sequence obtained by PCR amplification using the spl2 primers. The new splink2 sequence is shown in dark green, with the splink1 sequence (blue) at the right hand end. The C75R.2 primers were designed from sequence obtained by PCR amplification using the C75R.GSP primers. The new splinkC75R sequence is shown in turquoise and the C75R sequence in green.

3.2.2.5 Splinkerette PCR Using C75R2 Primers

In order to extend the sequence to the right of the splinkC75R sequence towards the terminal repeats, gene specific primers were designed against the splinkC75R sequence. These were named C75R2.GSP and C75R2.GSP.Int (Appendix 3)(Figure 3.2.2.4). The 5' end of the C75R2.GSP.Int primer was located 29bp to the right of the splinkerette ligation point and the 5' end of the C75R2.GSP primer was located 78bp to the right of the splinkerette ligation point. PCR amplification was performed using the C75R2.GSP primer and the splinkerette primer. The templates used were the 4 ligated OvHV-2 digests (*Hin*dIII, *Bam*HI, *Xba*I, *Eco*RI) as were used for PCR with the spl3 primers. PCR using this primer pair and repeating the first product as a template, using the internal primers, did not result in any product.

3.2.2.6 Design of New Splinkerette Linkers

Splinkerette PCR using the C75R2.GSP and Spl3.GSP primer resulted in no new products. It was thought that this could be due to the fact that the sequence at the terminal repeats was likely to be GC rich and the there were not many of the restriction sites required in this area. New splinkerette linkers were therefore designed requiring the digestion of OvHV-2 DNA restriction enzymes that recognise GC rich sites. These enzymes were *Xma*I, *Sal*I, *Nhe*I, *Bss*HII and *Bgl*II. OvHV-2 DNA was digested with these enzymes and ligated to splinker molecules as before. PCR amplification was performed using the splinkerette primer with the C75R2.GSP primer, and with the Spl3.GSP primer. The 5 new ligated OvHV-2 DNA samples were used as templates. The products of the initial amplification reaction were used as templates for PCR using the internal primers. Small products of approximately 150bp resulted from PCR amplification with C75R2.GSP internal primers using the *Nhe*I and *Sal*I digested DNA as template, and product of a similar size resulted from PCR with Spl3 internal primers using the *Xma*I digested DNA as template. The Spl3 primer product was lost on PCR purification and repeat of the PCR failed to reproduce this product. The product from PCR of the *Nhe*I and *Sal*I digested and ligated DNA was cloned into the TA vector. Positive colonies were selected and plasmid DNA purified. This plasmid DNA was analysed by restriction enzyme

digestion, however all plasmids were found to contain no insert. It was therefore assumed that all 3 products were primer dimers.

3.2.2.7 PCR Amplification Across the Viral Terminal Repeats

The last round of splinkerette PCR failed to result in new virus sequence, however, it was estimated that by comparison with the layout of the AlHV-1 genome, the distance to the terminal repeat sequences may be as little as 1kb at each end of the genome. Gammaherpesviruses contain variable numbers of copies of terminal repeats, therefore it was decided to try obtain the remaining OvHV-2 genome sequence by PCR amplification across the viral terminal repeat sequence using primers designed against the known sequence at the left and right hand ends of the genome.

It was hypothesised that the distance across may be up to 20 – 30kb, therefore an enzyme designed for long distance PCR was used. Expand long template PCR system uses an enzyme mix containing thermostable *Taq* DNA polymerase and a proofreading polymerase. It is designed to give a high yield of product from episomal and genomic DNA. Denaturation times were kept short (10s) and temperatures low (92°C) to avoid damage to the template DNA. Primers used (Figure 3.2.1.4 and Appendix 2) were longer than standard to permit the use of higher annealing temperatures which would enhance specificity.

The primers used were designed against the sequence derived by the second round of splinkerette PCR and were named C75R2long and spl3long. The primer spl3 long was the same primer as spl3 primer. The primer C75R2long was designed against the same coordinates as the primer splC75R2, however the sequence was the complement of splC75R2. The template used was genomic DNA extracted from the bovine OvHV-2 infected cell line BJ/1035. This cell line had been shown by Gardella gel analysis to contain OvHV-2 DNA in the circular episomal form. This DNA was therefore this was considered to be a suitable template as the terminal repeats would be fused, allowing PCR amplification across the repeats.

The PCR reaction was performed initially as described (Section 2.4.3). The enzyme used was the Expand Long Template PCR System (Roche). The reaction mix contained Expand PCR buffer 3 which resulted in a final magnesium

concentration of 2.25mM in each reaction. The annealing temperature used was 65°C and the elongation temperature used was 68°C as was recommended for the Expand enzyme. The initial extension time used was 20 minutes. This was increased by 20s each cycle until the extension time was 25 minutes, however the reaction did not result in any product. The PCR amplification reaction was repeated with a reduced annealing temperature of 62°C, and an extension time of 25 minutes extending by 20s each cycle to 30 minutes. Each reaction mix contained either an additional 1.5μl 50mM $MgCl_2$, to give a final concentration of 3.75mM, or an additional 2.5μl 50mM $MgCl_2$, to give a final reaction concentration of 5mM. These reactions did not result in the generation of any product.

Amplification of the first PCR product using internal primers, a magnesium concentration of 3.75mM and the longer extension time and reduced annealing temperature protocol, produced a high molecular weight smear. The PCR was again repeated using the high molecular weight product as template and internal primers. The products of this PCR reaction were a number of bands, the largest of which was approximately 1.3kb. A band of size 1.3kb and a band of size 650bp band were cloned using the pCR2.1 TOPO TA cloning kit. The clones were sequenced, however sequence analysis showed that the clones did not overlap with the right or left hand ends of the virus genome and had homology to cellular DNA sequences. It was concluded therefore, that the sequence was amplified from bovine cellular DNA, by mis-priming of the primers.

3.2.3 Sequence Analysis of the OvHV-2 Genome

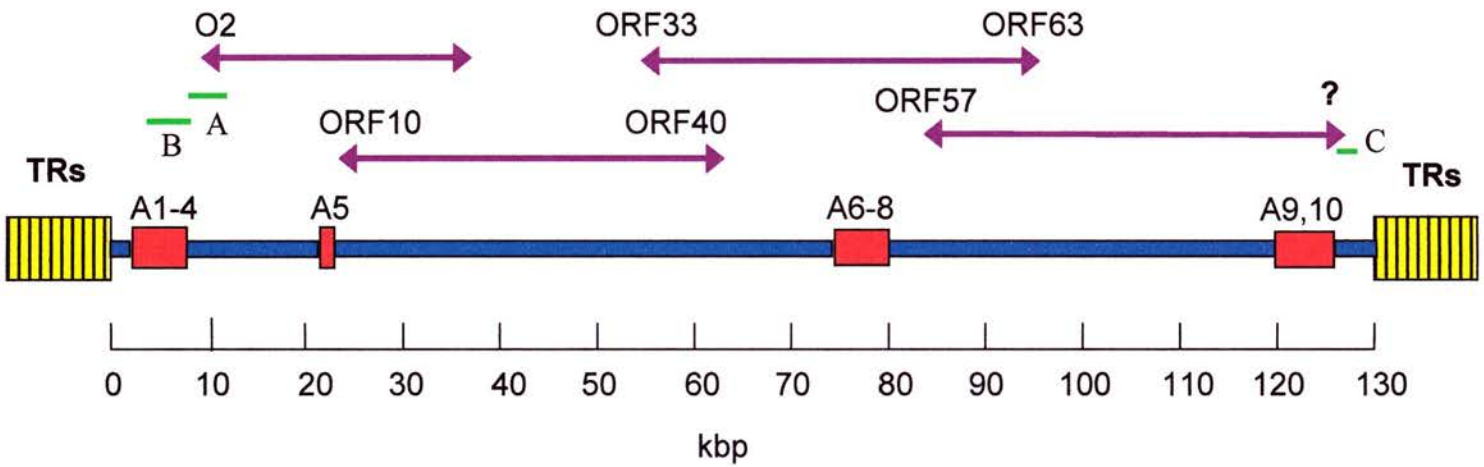
3.2.3.1 General Sequence Analysis

Cosmid cloning of the OvHV-2 genome resulted in the cloning of the majority of the OvHV-2 genome into five overlapping cosmids (Figure 3.2.3.1). Extending of the sequence by splinkerette PCR walking resulted in two new clones containing virus sequence at the left hand end of the genome and one new clone at the right hand end of the genome. The OvHV-2 genome sequenced so far is approximately 125kb in size. Sequencing of the cosmids was performed by Mathias Ackermann and co-workers at the University of Zürich, Switzerland. Sequence analysis of the genome was performed and the open reading frames identified using the NCBI ORF finder (Section 2.6.1). Potential protein coding open reading frames were defined by criteria similar to those applied to other herpesvirus genomes, these were:

1. ORF size larger than 60 amino acids
2. The presence of potential transcriptional start sites and polyadenylation sites
3. Homology to previously described genes in herpesviruses or other genes

The predicted open reading frames identified are listed in table 3.1. The translated sequences were analysed by comparison with the NCBI protein database using the program blastp. The amino acid identity with AlHV-1 ranges from 28% (ORF O6) to 83% (capsid protein vp23 encoded by ORF 26 and the minor capsid protein, encoded by ORF 43). The amino acid identity with herpesvirus saimiri ranges from 21% (ORF 10) to 58% (terminase exon 1, encoded by ORF 29a). The genome arrangement is very similar to that of AlHV-1, however there are notable differences. The layout of the predicted OvHV-2 open reading frames is shown in figure 3.2.3.2. Open reading frames with homologues in HVS have been numbered according to the HVS homologue. The genes without homologues in HVS have been named the "O" genes. The AlHV-1 "unique" genes A2, A3, A5, A6, A7, A8 and A9 have homologues in OvHV-2. The block of ORFs from ORF6 to 9 are all highly conserved and encode genes involved in viral DNA replication. The O2 ORF is predicted to be spliced, as is A2 of AlHV-1. This ORF contains leucine zipper motifs suggesting it may function in transcriptional regulation. O3 is a semaphorin

Figure 3.2.3.1 Location of the Ovine Herpesvirus 2 Cosmid Clones Against the AlHV-1 Genome



The location of the OvHV-2 cosmids are shown on the linearised genome of AlHV-1. The viral terminal repeats (TR) are shown in yellow. The position of the AlHV-1 unique genes are shown in red. The cosmids are shown in purple with the identity of the sequence at the ends marked. Where the sequence at the ends is not similar to any known open reading frames, a question mark is shown. The two clones obtained by splinkerette PCR are shown as green lines. The product Splink1 is marked "A", the product splink2 is marked "B" and the product splink C75R is marked "C".

Table 3.1 OvHV-2 ORFs

OvHV-2 ORF	*Strand*	*Start Codon*	*Stop Codon*	*Length (aa)*	*AlHV-1 % identity (similarity)*	*HVS % identity (similarity)*	*Possible Function*
O2b	-	1990	1841				
O2a	-	1746	1336	186	56(71)		Leucine zipper motifs
O2.5a	+	2753	2938	182			Il-10 homologue
O2.5b	+	3021	3080				Signal sequence cleavage 26-27 aa
O2.5c	+	3162	3314				51% identity/69% sim. to Equine IL-10
O2.5d	+	3406	3471				
O2.5e	+	3552	3632				
O3	+	3978	5354	458	50(63)		Semaphorin (NB shorter by 200aa than A3 at C-terminus)
O3.5	+	6088	6579	163			Signal peptide: secreted
3	+	6831	10916	1361	52(57)	28(46)	FGARAT
O4.5	+	10972	11610	212	50(73)		Bcl-2 homologue ? viral Bcl-2 antagonist similar to EBV BALF1
6	+	11752	15141	1129	79(88)	50(69)	Single-stranded DNA binding protein
7	+	15216	17264	682	65(79)	41(60)	Transport protein
8	+	17248	19839	863	76(84)	44(62)	Glycoprotein B
9	+	20034	23030	998	75(86)	56(70)	DNA polymerase
O5	+	23312	24565	417	49(72)		GPCR. NB has much longer C-terminal intracellular tail than A5
10	+	24285	25505	406	56(72)	21(40)	
11	+	25507	26739	410	56(75)	30(49)	
17	-	37734	36076	552	50(61)	34(47)	Capsid Protein
18	+	37691	38521	276	68(79)	45(65)	
19	-	40174	38495	559	74(85)	41(58)	Tegument protein
20	-	40715	39963	250	61(72)	41(58)	Fusion protein
21	+	40714	42423	569	47(63)	29(47)	Thymidine kinase
22	+	42444	44696	750	63(78)	27(47)	Glycoprotein H
23	-	45891	44689	400	58(71)	34(53)	
24	-	48086	45897	729	70(80)	41(59)	
25	+	48085	52188	1367	81(91)	57(74)	Major capsid protein
26	+	52254	53168	304	83(92)	48(67)	Capsid protein vp23
27	+	53174	54055	293	51(69)	28(41)	
29b	-	55287	54142	352	82(90)	58(78)	Terminase exon 2
30	+	55305	55556	83	57(73)	33(50)	
31	+	55478	56152	224	67(75)	41(59)	
32	+	56101	57531	476	48(61)	25(44)	
33	+	57521	58540	339	52(67)	37(54)	
29a	-	59447	58530	328	76(85)	53(67)	Terminase exon 1
34	+	59446	60459	337	63(75)	37(58)	
35	+	60437	60898	153	59(76)	29(52)	
36	+	60792	62114	440	55(70)	33(52)	Phosphotransferase
37	+	62098	63555	485	79(88)	42(63)	Alkaline exonuclease
38	+	63510	63695	61	49(67)	47(64)	Myristylated tegument protein
39	-	64967	63741	408	80(92)	44(67)	Glycoprotein M
40	+	65011	67038	675	48(64)	26(40)	Helicase-primase
42	-	67808	67035	257	64(78)	36(59)	
43	-	69468	67780	562	83(92)	54(70)	Minor capsid protein

OvHV-2 ORF	*Strand*	*Start Codon*	*Stop Codon*	*Length (aa)*	*AlHV-1 % identity (similarity)*	*HVS % identity (similarity)*	*Possible Function*
44	+	69323	71815	830	78(88)	55(72)	Helicase
45	-	72656	71871	261	37(47)	22(38)	
46	-	73552	72689	287	71(87)	56(71)	Uracil DNA glycosylase
47	-	73883	73428	151	45(57)	28(48)	Glycoprotein L
48	-	75278	73938	446	41(57)	25(49)	
50a	+	75449	75505	573	36(51)	22(39)	Transcriptional control – RTA exon 1
49	-	76520	75544	324	N/A	23(41)	
50b	+	76556	78220				Transcriptional control – RTA exon 2
O6a	+	78532	79056	256	28(46)	N/A	Viral Transactivator BZLF1 homologue
O6b	+	79166	79270				
O6c	+	79366	79503				
O7	+	80078	80302	556	60(79)	N/A	Virion Glycoprotein exon1
O8a		80691	81933		41(56)	N/A	Exon2
O8b		82881	83080				Exon3
52	-	83552	83142	136	45(68)	26(44)	
53	-	83942	83634	102	51(72)	35(59)	Glycoprotein N
54	+	84011	84892	293	57(75)	36(54)	dUTPase
55	-	85592	84936	218	74(86)	43(67)	
56	+	85591	88104	837	67(80)	43(59)	Helicase-primase complex
57a	+	88237	88288	433	51(67)	23(39)	Transcriptional control MTA exon 1
57b	+	88391	89640				Transcriptional control MTA exon 2
58	-	91147	90092	351	60(77)	26(46)	
59	-	92323	91154	389	63(72)	33(50)	Processivity factor of DNA pol
60	-	93372	92455	305	80(89)	57(76)	Ribonucleotide reductase (small subunit)
61	-	95781	93424	785	71(82)	51(67)	Ribonucleotide reductase (large subunit)
62	-	96819	95812	335	63(79)	32(52)	Capsid assembly and maturation
63	+	96818	99661	947	61(77)	27(47)	Tegument protein
64	+	99666	107540	2624	50(66)	24(40)	Large tegument protein
65	-	108240	107605	211	41(49)		Capsid protein
66	-	109604	108297	435	60(73)	36(54)	
67	-	110299	109523	258	68(79)	51(74)	Virion tegument protein
67a	-	110561	110307	84	69(86)	48(72)	
68	+	110781	112199	472	61(76)	39(54)	Probable major envelope glycoprotein
69	+	112206	113060	284	74(88)	44(61)	
O8.5	+	116954	118126	390			
73	-	119710	118223	495	43(48)		LANA homologue; episomal maintenance
75	-	124231	120281	1316	58(76)	29(46)	FGARAT
O9	+	124613	125233	206	50(69)		Bcl-2 homologue

Key: Red. Homologous to AlHV-1 unique A gene
Pink. Unique O gene

Figure 3.2.3.2 Genome Organisation of Ovine Herpesvirus 2

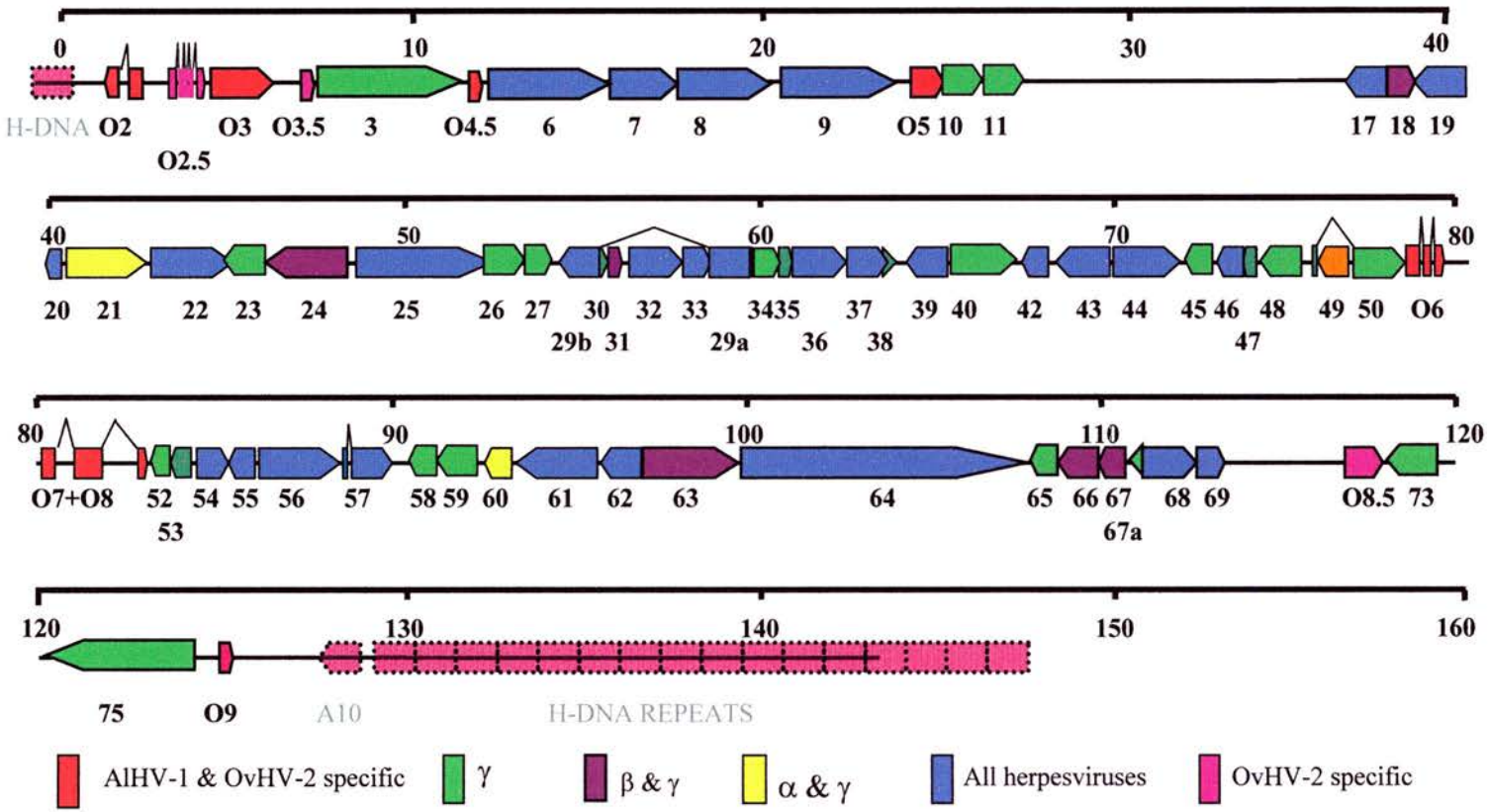


Figure 3.2.3.2 The Genome Organisation of OvHV-2. The diagram shows the linearised genome sequence of OvHV-2 that was cloned into cosmids. The open reading frames are represented as block arrows, which are coloured according to the conservation of the ORF among the three subfamilies (α, ß and γ) of herpesviruses. The ORFs unique to OvHV-2 and AlHV-1 are shown in red, whilst the ORFs unique to OvHV-2 only are shown in pink. The genome length is shown in kbp. ORF 49, shown in orange, is missing in AlHV-1 but is present in HVS, PLHV-1 and other gammaherpesviruses. The sequence not yet cloned, but predicted to be present by comparison with the AlHV-1 genome sequence, is shown in semi-transparent colour. This diagram was based on the diagram of the AlHV-1 ORFs shown in (Ensser *et al.*, 1997).

homologue, and contains a highly conserved "sema" domain. O5 is predicted to be a G protein-coupled receptor (GPCR), with homology to A5 of AlHV-1, A5 of PLHV-1 and to the EHV-2 gene E6. This is the only GPCR homologue as, in common with AlHV-1, OvHV-2 does not encode an ORF 74 homologue. The ORFs O6, O7, and O8 have homologues in AlHV-1 and in the porcine gammaherpesvirus PLHV-1. The O6 ORF is predicted to be spliced into 3 exons and is positionally equivalent to the EBV transactivator BZLF1. Transcriptional analysis of the PLHV-1 A6 gene has shown that the transcript is spliced into 3 exons in the same way as is predicted for the homologue in OvHV-2. O7 and O8 are predicted to form two exons of a putative glycoprotein similar in function to gp350 of EBV. The O8 ORF is also predicted to be spliced. The O9 ORF is predicted to encode a bcl-2 homologue and has 50% amino acid identity to the bcl-2 homologue A9 of AlHV-1.

OvHV-2 contains two copies of the putative viral tegument protein with homology to the purine biosynthetic enzyme formylglycineamide ribotide amino transferase (FGARAT) in different orientations, located at each end of the genome at ORF 3 and ORF 75. These conserved genes are also present in AlHV-1, HVS, and BoHV-4, however only one copy of the FGARAT gene is found in KSHV and EBV. ORF 3 of PLHV-1 is also a FGARAT homologue (Goltz *et al.*, 2002), however the area of ORF 75 has not yet been sequenced.

The ORF O4.5 encodes a 212 aa protein with homology to bcl-2. Homologues of this protein are found in EHV-2 (E4), PLHV-1, in EBV (BALF1) and in AlHV-1. This means that OvHV-2 has two putative bcl-2 homologues (located at O4.5 and at O9), as does EBV (BALF1 and BHRF1) and AlHV-1. Analysis of the translated sequence using the blastp program showed the O4.5 sequence had 46% amino acid identity with the BALF1 homologue of AlHV-1. The sequence also had 32% amino acid identity to the PLHV-1 BALF1 homologue and 22% identity to the E4 sequence of EHV-2. This method of sequence analysis did not reveal homology with any bcl-2 protein sequences.

The translated ORF O4.5 sequence was compared to other viral and cellular bcl-2 homologues by ClustalW analysis (Figure 3.2.3.3). The conserved BH domains were located on the sequence by comparison with the location in other bcl-2 sequences. The analysis showed that although O4.5 was most similar to the AlHV-1

Figure 3.2.3.3 Alignment of Viral and Cellular Bcl-2 Sequences

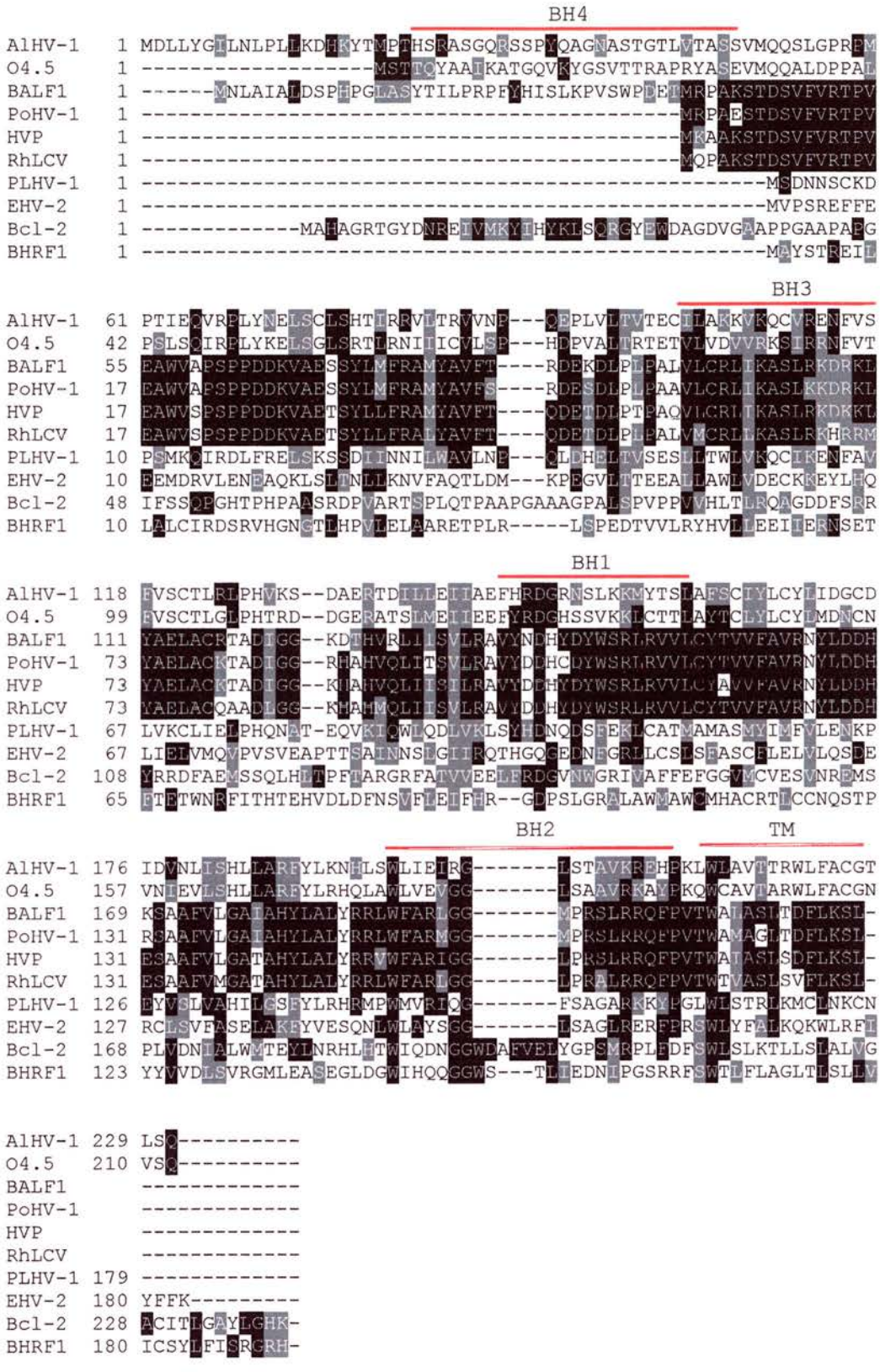


Figure 3.2.3.3 Alignment of viral and cellular bcl-2 sequences. The amino acid sequences of viral and cellular bcl-2 homologues (AlHV-1 BALF1, OvHV-2 O4.5, EBV BALF1, Pongine herpesvirus 1 (PoHV-1) BALF 1, herpesvirus papio (HVP) BALF1, rhesus lymphocryptovirus (RhLCV) BALF1, PLHV-1 BALF1, EHV-2 E4, human bcl-2, EBV BHRF1) were aligned using the Blosum scoring matrix with the "ClustalW" program (see 2.6.2), and displayed using the "BOXSHADE" program. Identical residues are highlighted in black, whilst similar residues are highlighted in grey. The position of the bcl-2 homology (BH) domains and C terminal transmembrane hydrophobic region (TM) are marked above the alignment in red.

BALF1 homologue, there were amino acids conserved in all the sequences, especially in the areas of the predicted BH domains. The OvHV-2 BALF1 homologue appeared to encode all four bcl-2 homology (BH) domains, although the BH4 domain was less well conserved than the other domains. The BALF1 homologous sequences from the primate gammaherpesviruses were highly conserved, probably reflecting similarity in the host species. All BALF1 homologous sequences were more highly related to each other than to the BHRF1 or bcl-2 sequences, suggesting that these proteins are a separate group of bcl-2 like proteins.

3.2.3.2 Open Reading Frames Present in OvHV-2 But Not in AlHV-1

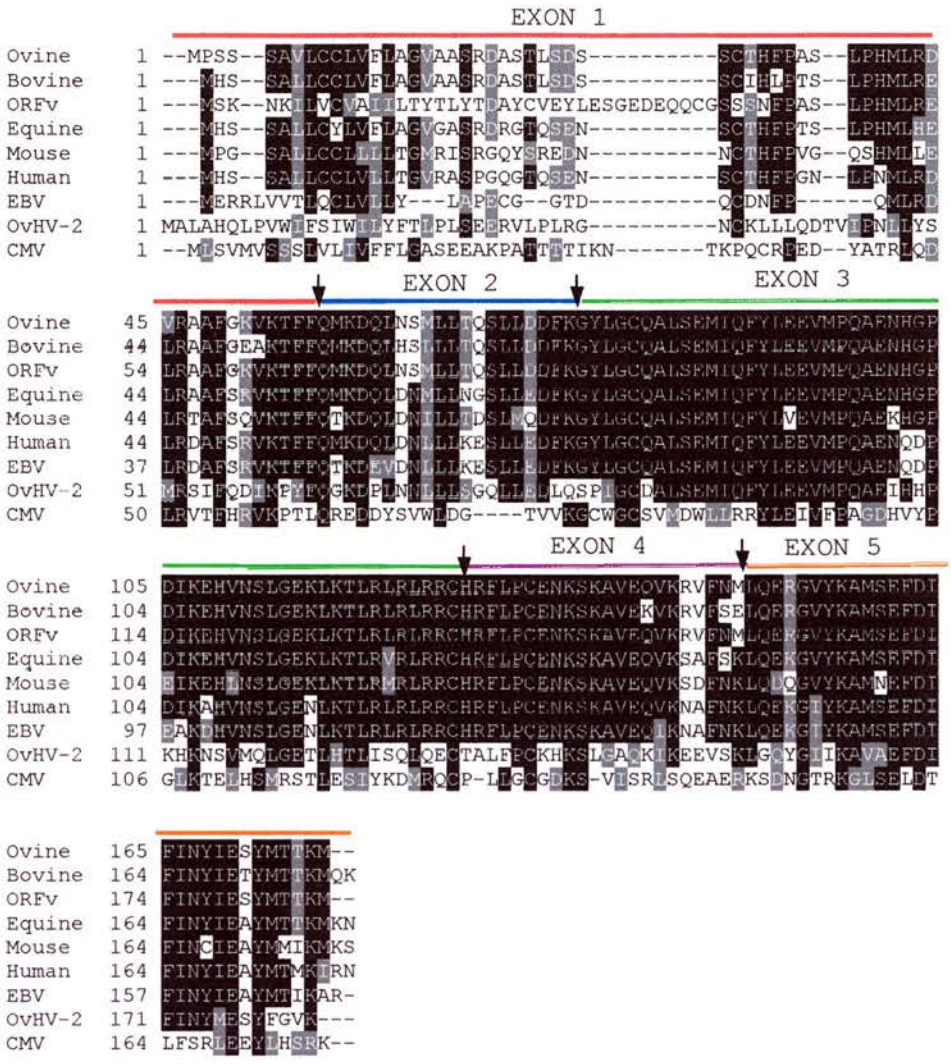
The open reading frames present in OvHV-2 but not in AlHV-1 are of great interest as they confirm OvHV-2 as a gammaherpesvirus that is distinct from AlHV-1. The open reading frame ORF49 is present in OvHV-2 but is missing from AlHV-1. This ORF is not OvHV-2 specific, however as it is present in other gammaherpesviruses including HVS and also in the closely related PLHV-1. The open reading frame O3.5 is in the same position as A4 of AlHV-1. It is predicted to encode a 163 aa secreted protein and contains a predicted signal peptide sequence, however the ORF is not homologous to A4 of AlHV-1. The O2.5 open reading frame does not have a positional homologue in AlHV-1; this ORF encodes a 182 aa protein with homology to IL-10. IL-10 homologues are also present in EBV (BCRF1) and in EHV-2 (ORF E7) sequence. Analysis of the OvHV-2 ORF O2.5 translated sequence shows that the sequence is highly homologous to a large number of IL-10 sequences (Table 3.2). The OvHV-2 IL-10 homologue is different from E7 and BCRF1 in that it contains potential splice donor and acceptor sites such that it is predicted to be spliced into 5 exons. ClustalW analysis of the translated IL-10 sequence (Figure 3.2.3.4) illustrated the similarity of both viral and cellular IL-10 sequences to each other. The OvHV-2 IL-10 sequence seemed to be more distantly related to the cellular IL-10 sequences than the ORF virus IL-10 or the EBV IL-10. Comparison of the predicted splice sites with the splice sites found in the human IL-10 sequence revealed that the predicted splice sites in the OvHV-2 sequence were at equivalent sites, as shown on the ClustalW diagram. This suggests that the splice sites predicted might to be used *in*

vivo. The O8.5 ORF encodes a 390 amino acid protein that has no homologues in other herpesviruses. The possible function of this sequence is not known.

Table 3.2 Results from blastp analysis of the translated sequence of OvHV-2 ORF O2.5.

IL-10 Source	% Identity	% Similarity
Equine	45	60
EHV-2	44	60
Saimiri sciureus	44	59
Murine	42	58
Rat	42	58
Human	45	59
Bovine	45	59
Red Deer	45	60
Ovine	44	59
ORF virus	44	59
Porcine	42	58

Figure 3.2.3.4 Alignment of Viral and Cellular IL-10 Sequences



The amino acid sequences of viral and cellular IL-10 amino acid sequences (Ovine, bovine, ORF virus (ORFv), equine, mouse, human, Epstein-Barr virus (EBV), Ovine herpesvirus-2 (OvHV-2), human cytomegalovirus (CMV)) were aligned using the Blosum scoring matrix with the "ClustalW" program, and displayed using the "BOXSHADE" program (see 2.6.2). Residues that were identical are highlighted in black, whilst similar residues are highlighted in grey. The positions of the five exons in cellular sequences are shown as coloured lines, with arrows at the boundary between exons.

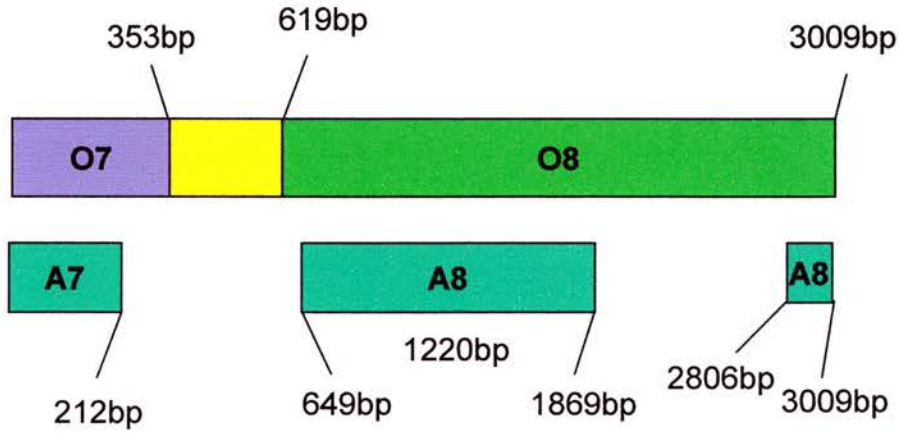
3.3 Characterisation of OvHV-2 O7 and O8

Cosmid cloning of the OvHV-2 genome resulted in cloning of a large proportion of the virus genome, therefore the aim was to study an area of the genome in more detail. A non-sequence conserved glycoprotein gene is located in the centre of the genome of all gammaherpesvirus genomes sequenced so far (Birkmann *et al.*, 2001). In EBV this is the major envelope glycoprotein gp350/220, which mediates binding of the virus to the B lymphocyte receptor CD21. Virus neutralising antibodies can be raised against gp350/220; therefore the protein has been used in vaccine studies. An equivalent protein, gp150 of MHV-68, has been shown to bind specifically to lymphocytes (I. Atkin, unpublished). OvHV-2 equivalent ORFs are O7 and O8 and are located near the centre of the linearised genome map, along with O6 (Figure 3.2.3.1). It was decided to study the predicted open reading frames O7 and O8, which were contained in the cosmid C57 sequence. By analogy with gp350, O7 and O8 may function as a viral glycoprotein involved in viral entry into target cells. The aim of this study was to further characterise O7 and O8, in order to gain some insight into the possible function of this protein in viral pathogenesis.

3.3.1 Initial Characterisation of O7 and O8

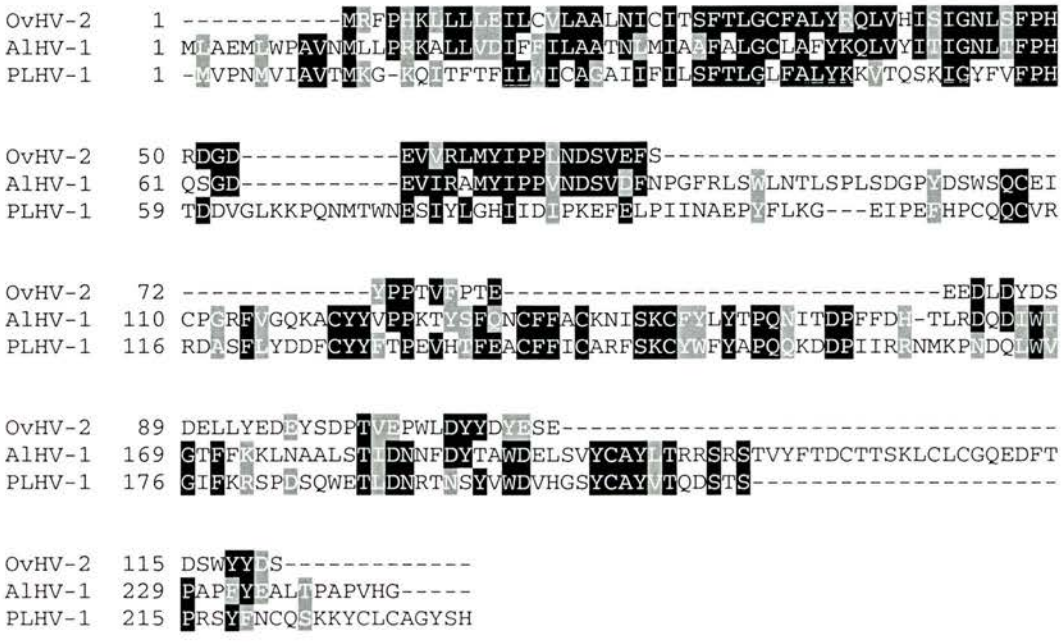
The predicted O7 and O8 amino acid sequences were initially analysed by comparison with the protein database using the translated BLAST search tblastx, as described (Section 2.6.1). O7 and O8 have the highest homology with the AlHV-1 sequence. O7 shares 60% amino acid identity with A7 of AlHV-1. The similarity is located in one distinct region from nucleotides 1 – 212, relative to the ATG start codon at the beginning of the O7 ORF (Figure 3.3.1.1). The predicted O7 open reading frame is 121 aa in length. This is considerably shorter than the O7 of AlHV-1 (243 aa) or PLHV-1 (234 aa). O8 shares on average 56% amino acid identity with A8 of AlHV-1. The homologous areas of O8 are located in two distinct blocks, from nucleotides 643 –1863, and from 2892 – 3002. O8 also displays homology with sequence of PLHV-1, with 20% amino acid identity. The translated sequences of AlHV-1, OvHV-2 and PLHV-1 were aligned for comparison using the ClustalW

Figure 3.3.1.1 Sequence Analysis - Areas of O7/O8 Homologous to A7/A8



The O7 and O8 sequence was analysed using the translated blast search tblastx. The diagram shows the region encoding the O7 and O8 open reading frames. The predicted O7 open reading frame is shown in lilac, and the predicted O8 open reading frame is shown in green. The region between the O7 and O8 sequences is shown in yellow. The regions of the translated O7 and O8 sequence homologous to AlHV-1 are shown as turquoise blocks below the O7 and O8 sequence.

Figure 3.3.1.2 Alignment of A7 Amino Acid Sequences.



The A7 amino acid sequences of OvHV-2, AlHV-1 and PLHV-1 were aligned using the Blosum scoring matrix with the "ClustalW" program, and displayed using the "BOXSHADE" program. Identical residues in two or more sequences were highlighted in black, while similar residues were highlighted in grey.

Figure 3.3.1.3 Alignment of A8 Amino Acid Sequences

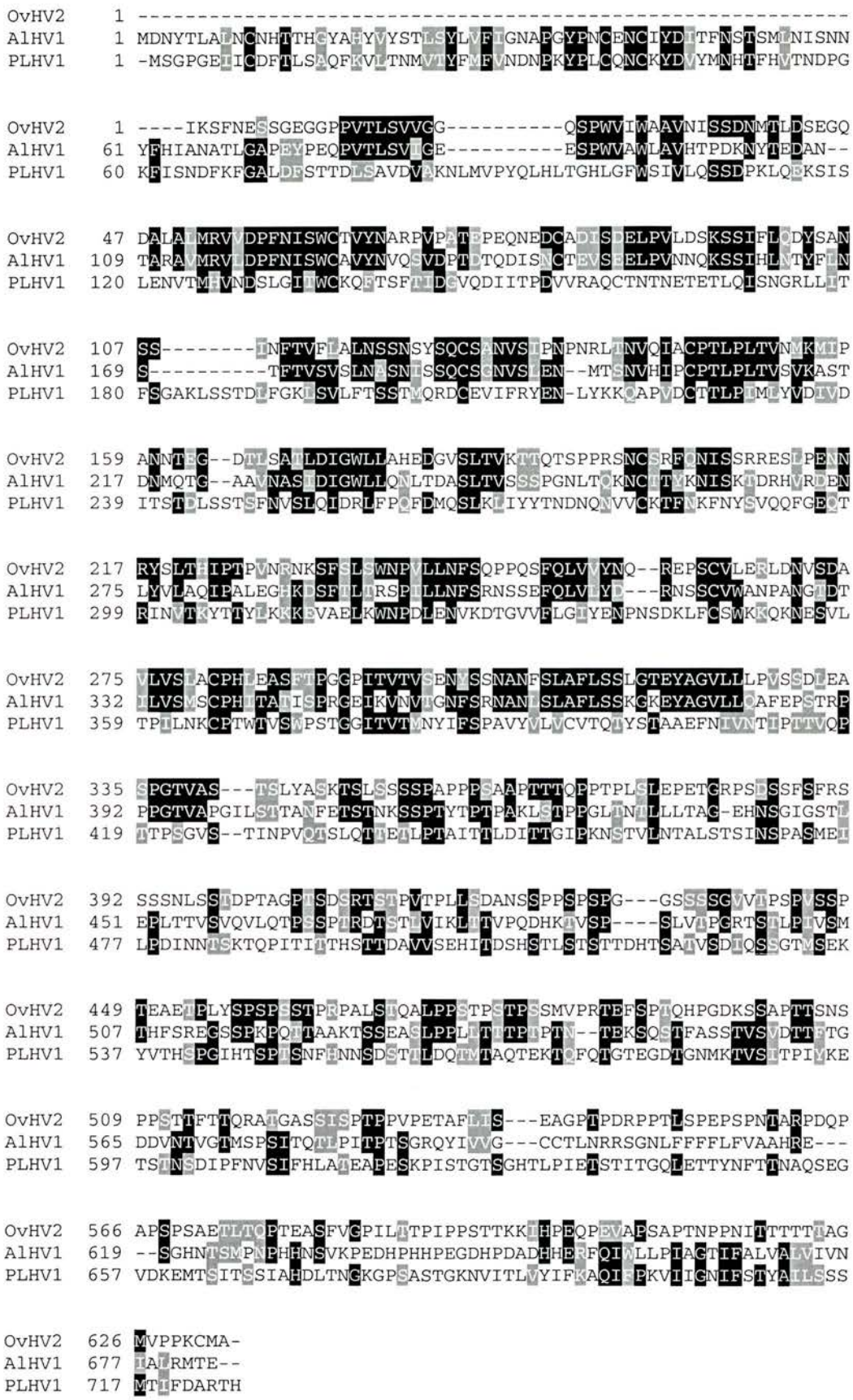


Figure 3.3.1.3 Alignment of A8 homologous amino acid sequences. The amino acid sequences of the A8 homologues of AlHV-1, PLHV-1 and OvHV-2 were aligned using the Blosum scoring matrix with the "ClustalW" program (Section 2.6.2), and displayed using the "BOXSHADE" program. Identical residues are highlighted in black, whilst similar residues are highlighted in grey.

program (Section 2.6.2) and diagrams of the similarities for both O7 and O8 made using the boxshade program (Figure 3.3.1.2 & 3). It can be seen that the greatest similarity is between the OvHV-2 and the AlHV-1 sequences, however some amino acids are conserved between all three proteins. Possible promoter sequences for O7/O8 can be seen at –74 (TATATTG) and at –55 (TATTTAAA) relative to the translational start site of O7. A putative poly A signal (AATTAAA) can be seen 45bp downstream of the O8 stop codon. This work was based on an initial hypothesis that O7 and O8 encode separate domains of the same glycoprotein. The O7 and O8 sequence was therefore translated into a continuous 844 aa sequence encoding both O7 and O8 using identified splice sites that it was considered were the most likely to be used in translation of an O7 and O8 protein (discussed later). This sequence was was used for subsequent protein sequence analysis.

Virion membrane glycoproteins such as gp350 and gp150 are type I membrane glycoproteins that contain N and O linked glycosylation, an N terminal hydrophobic signal sequence and a C terminal hydrophobic membrane anchor. Sequence analysis of O7/O8 was performed to identify predicted features in common with other virion membrane glycoproteins. Initial analysis revealed the presence of N-linked glycosylation motifs in both the predicted O7 and O8 open reading frames. Asparagine N-glycosylation sites were predicted using the NetNGlyc program (Gupta *et al.*, 2002) and possible serine and threonine O-linked glycosylation sites were predicted using the NetOglyc2.0 program (Hansen *et al.*, 1995). O7/O8 has 22 potential sites for N-glycosylation, shown diagrammatically on figure 3.3.1.4. O7/O8 has 51 potential serine O linked glycosylation sites, and 43 potential threonine O-linked glycosylation sites, all are shown diagrammatically on figure 3.3.1.5. The area from amino acids 500 – 770 is predicted to be highly O-glycosylated.

Hydropathicity analysis of the translated O7/O8 sequence was performed, using the method of (Kyte & Doolittle, 1982) (Section 2.6.2). This shows that the areas of the highest hydropathicity were located at the N terminus of the predicted O7 sequence and at the C terminus of the predicted O8 sequence. The sequence was also analysed using the program TMHMM2.0 (Sonnhammer *et al.*, 1998). This predicted that the N terminal hydrophobic region of O7 and C terminal hydrophobic

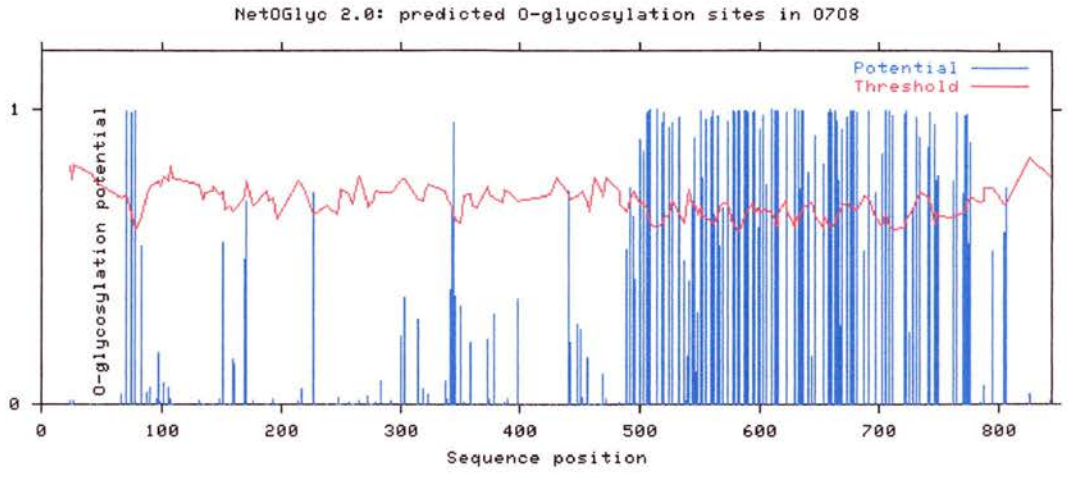


Figure 3.3.1.4 Potential O-Glycosylation sites in the O7/O8 sequence as predicted using the NetOGlyc program. The translated O7/O8 sequence is shown linearised on the horizonal axis with amino acids numbered from 0 – 844. The threshold for glycosylation is shown in red, and potential O-glycosylation sites in the sequence are shown as vertical blue lines extending above the height of the threshold line.

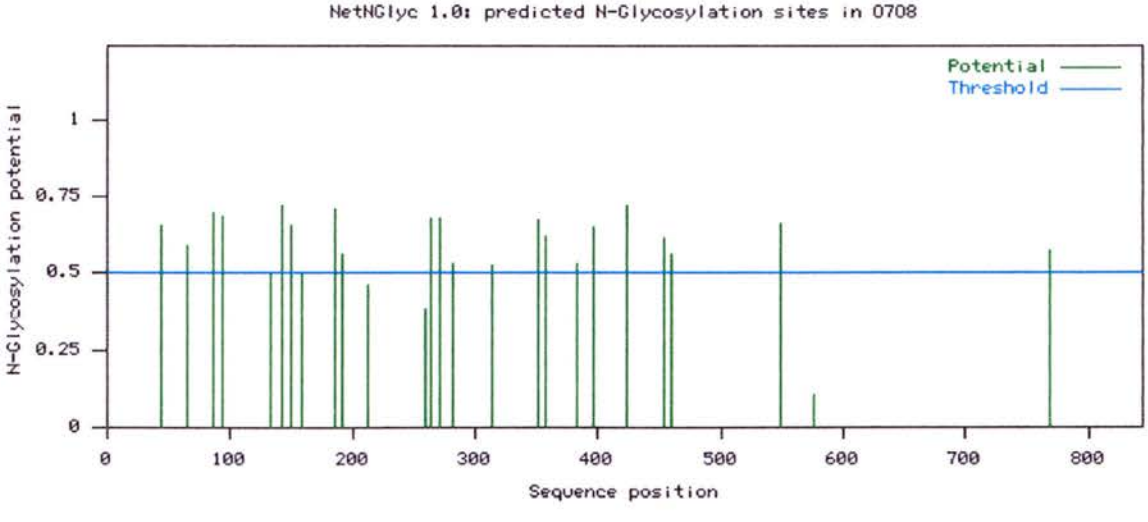


Figure 3.3.1.5 Potential N-Glycosylation sites in the O7/O8 sequence as predicted using the NetNGlyc program. The translated O7/O8 sequence is shown linearised on the horizontal axis, with the amino acids numbered from 0 – 844. The threshold for N-Glycosylation is shown as a horizontal blue line, and potential N-Glycosylation sites are shown as vertical green lines extending above the height of the threshold line.

region of O8 were likely to be transmembrane regions of the protein. The N terminal predicted transmembrane region extended from amino acid 7 to 29, with amino acid 1 to 6 predicted to be inside the membrane. The C terminal transmembrane region extended from amino acid 818 to 840 and could form a membrane anchor, with amino acids 841 – 844 being back inside the membrane (Figure 3.3.1.7).

It was suspected that the N terminal hydrophobic region of the protein might encode a signal peptide, therefore the program SignalP (Nielsen *et al.*, 1997) was used to predict the probability of the presence of a signal sequence in O7/O8, and the location of a possible signal cleavage site. The program predicted that the sequence from amino acids 1 – 32 was a signal peptide, with the signal cleavage site being between amino acids 32 and 33. The signalP program used the C-score (raw cleavage site score), the S-score (signal peptide score) and the Y score (combined cleavage site score) at each location of the sequence, to predict the location of the signal cleavage site. The C-score is high at position +1 after the cleavage site, and low at all other positions, the S-score is high at all positions before the cleavage site, and low at 30 positions after the cleavage site and in the N terminal regions of non-secretory proteins. The prediction of cleavage site location is optimised by observing where the C-score is high, and the S-score changes from a high to a low value. The Y score shows this by combining the height of the C-score with the slope of the S-score. A diagram of the C, S and Y scores for the O7/O8 sequence is shown on figure 3.3.1.8.

The fact that the predicted O7 amino acid sequence contained a hydrophobic signal sequence but no membrane anchor, and the O8 sequence was predicted to contain a hydrophobic membrane anchor but no signal sequence strongly suggested that these two ORFs might form one gene and be spliced together to produce one protein product. This was further suggested by the lack of any good TATA box sequence upstream of O8 and the lack of a poly A signal downstream of O7.

The nucleotide sequence from O7-O8 was analysed for the presence of predicted splice donor and acceptor sites using the NetGene2 program (Brunak *et al.*, 1991, Hebsgaard *et al.*, 1996). The location of the splice donor and acceptor sites is shown in figure 3.3.1.9. The O7 open reading frame runs continuously from nucleotides O – 363, with respect to the O7 translational start site and the O8 open

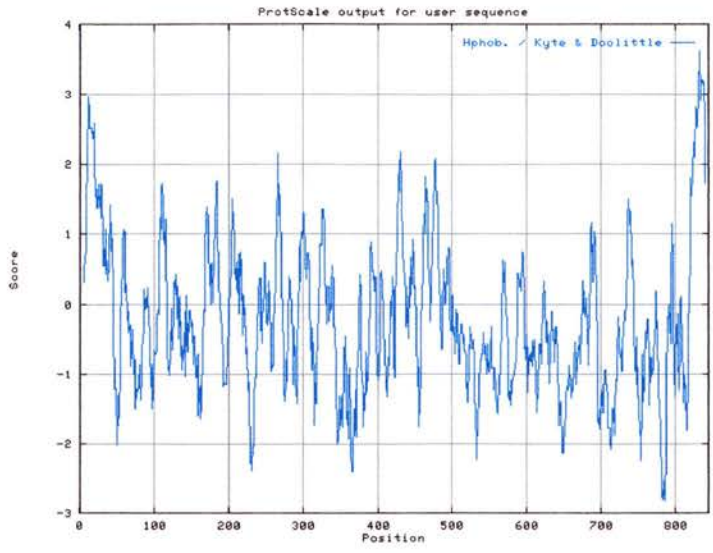


Figure 3.3.1.6 A hydropathicity plot of the O7/O8 translated sequence performed using the method of Kyte & Doolittle (1982). The translated O7/O8 sequence is shown linearised on the horizontal axis. Hydropathicity of each amino acid is shown plotted on the vertical axis. Hydrophobic regions of the sequence have positive values, whilst hydrophilic regions have negative values.

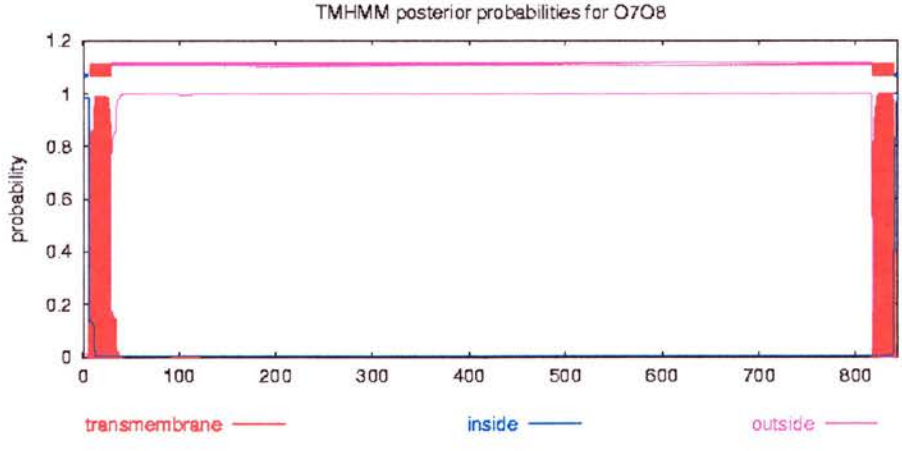
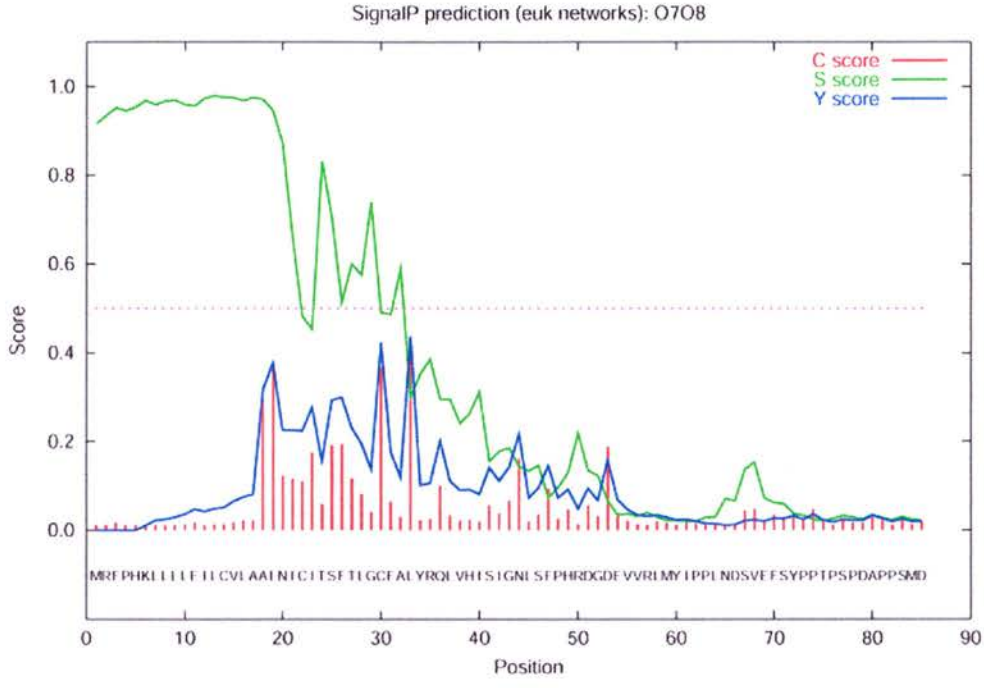


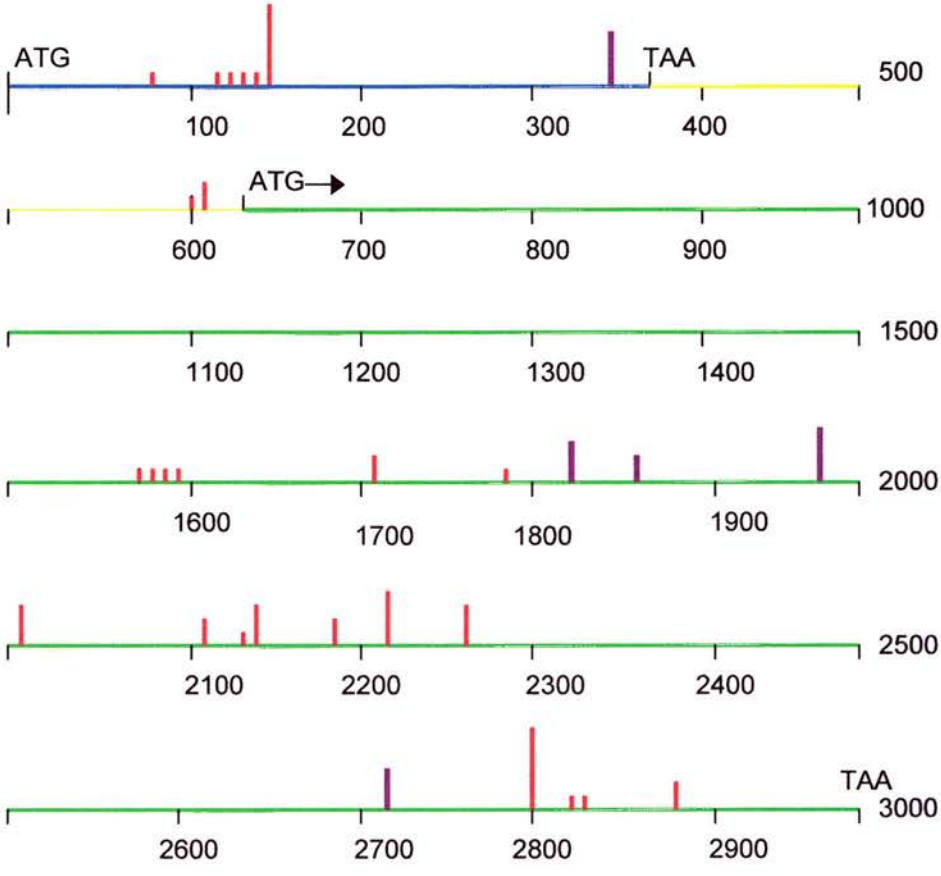
Figure 3.3.1.7 The diagram shows transmembrane helices in O7/O8, predicted using the program TMHMM2.0. The O7/O8 translated sequence is shown linearised on the horizontal axis. Transmembrane regions are shown in red, whilst areas on the outside of the membrane are shown in pink, and areas inside the membrane in blue. The program uses an algorithm called N-best to predict the most probable location of transmembrane helices in the sequence. In the area of probability between 1 and 1.2 the N-best prediction of the location of transmembrane helices is shown.

Figure 3.3.1.8 Prediction of Signal Cleavage sites in O7/O8



The diagram shows a graphical representation of the scores used in prediction of signal cleavage sites in the O7 sequence, using the program signal P. The first 90 amino acids of the translated O7/O8 are shown linearised on the horizontal axis. The C-score, S-score and Y-score as predicted by the program, are plotted for each amino acid on the vertical axis. The C-score is high at position +1 after the cleavage site, and low at all positions, the S-score is high at all positions before the cleavage site, and the Y score combines the height of the C-score with the slope of the S-score to predict the cleavage site, which for O7/O8 was located between position 32 and 33.

Figure 3.3.1.9 Prediction of Splice Donor And Acceptor Sites In O7/O8 Sequence Using The NetGene2 Programme



The diagram shows the location of splice donor and acceptor sites in the sequence encoding the O7 and O8 ORFs. Splice donor and acceptor sites in the sequence were predicted using the NetGene 2 programme. The predicted O7 open reading frame is shown in blue and the predicted O8 open reading frame is shown in green. The area between the open reading frames is shown in yellow. Translational start sites (ATG) and stop sites (TAA) are shown. The coordinates shown are nucleotide numbers relative to the O7 start site. The splice donor and acceptor sites are shown as vertical lines extending from the position of the site. The height of the line is proportional to the confidence value with with the programme predicted the site. Splice donors are shown in purple and splice acceptors are shown in red.

reading frame runs from 637 – 2720 (O8a), where a splice acceptor splices into a different frame to 2802. O8 then ends at 3002 (O8b). There are a number of predicted splice donor and acceptor sites in the two open reading frames, raising the possibility of alternative splicing of the sequence.

Analysis of the sequence using the ScanProsite program (Section 2.6.2) revealed a proline rich domain in the putative ectodomain region towards the membrane anchor (509 – 768), this feature is found in other herpesvirus glycoproteins such as gp150 of MHV-68 and gp350/220 of EBV (Stewart *et al.*, 1996). The sequence also contained a serine rich domain from amino acids 530 – 712, and a threonine rich domain from amino acids 557 – 816. The sequence was analysed for possible antigenic sites using the program ANTIGENIC, found on the EMBOSS web site (Section 2.6.1). Analysis identified 34 possible antigenic regions, which are shown in figure 3.3.1.10.

In summary, sequence analysis resulted in the hypothesis that O7 and O8 are spliced together to form a membrane glycoprotein. The signal peptide of the glycoprotein was predicted to be encoded by O7, whilst the C terminal region of O8 was predicted to form a membrane anchoring region. The sequence was predicted to be highly glycosylated and multiple splice variants were possible. These findings form a basis for experimental investigation of the properties and likely function of the O7/O8 region.

3.3.2 Analysis of The Expression of O7/O8 In OvHV-2 Infected Cell Lines

3.3.2.1 Northern Analysis

Analysis of the sequence of O7 and O8 predicted that the sequences were spliced together to form a glycoprotein. In order to confirm this, the expression of mRNA encoding O7 and O8 in rabbit cell lines was studied. Northern analysis for ORF 75 showed that expression of productive cycle genes in OvHV-2 infected rabbit cell lines is low, therefore efforts were made to increase the expression of productive cycle genes of OvHV-2. Treatment of KSHV infected BCP-1 cell lines with sodium butyrate has been shown to increase expression of productive cycle genes (Dr S.J Talbot, personal communication) therefore this approach was used for the OvHV-2 infected rabbit cell line BJ/880.

Figure 3.3.1.10 Antigenic sites of O7/O8

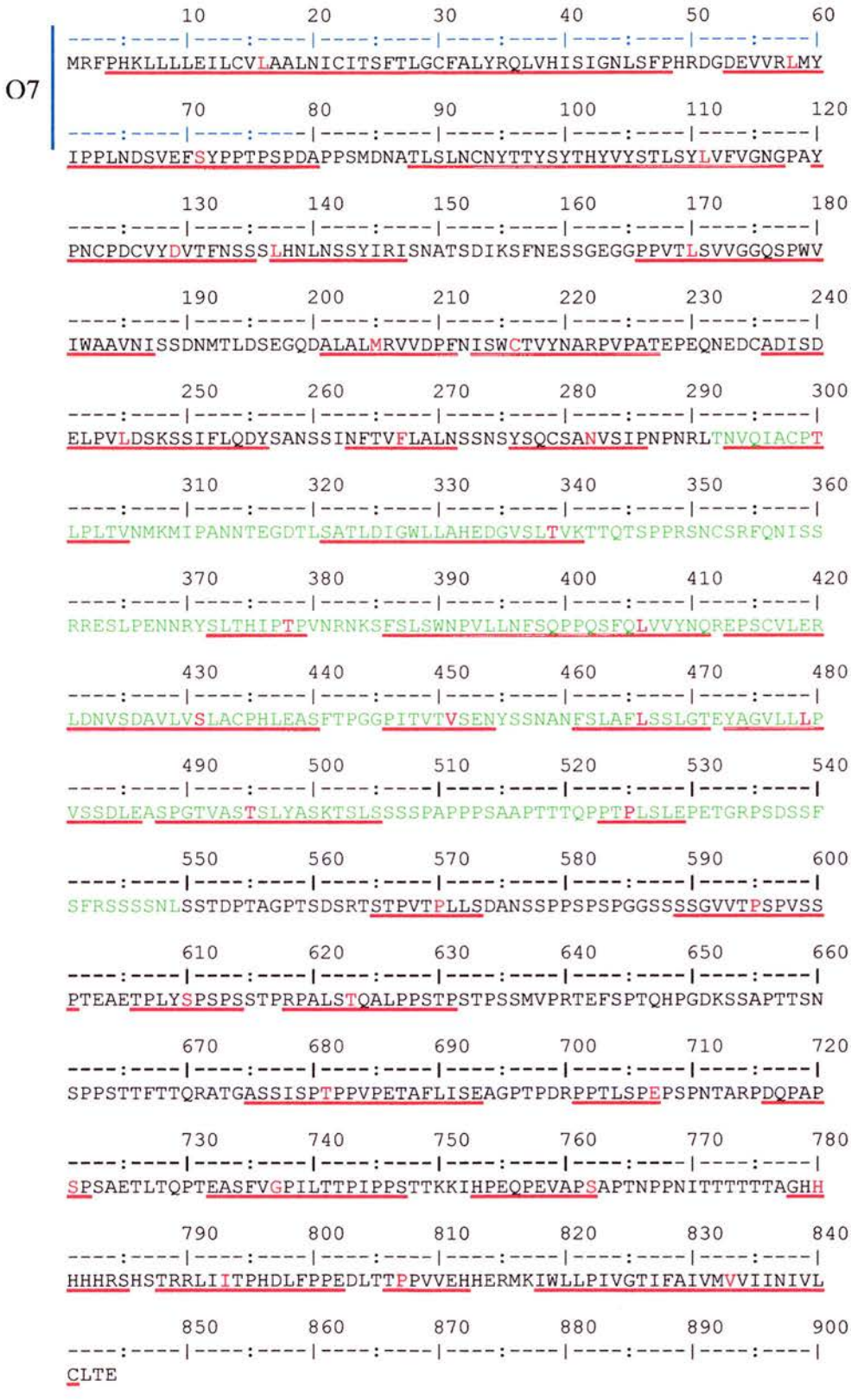
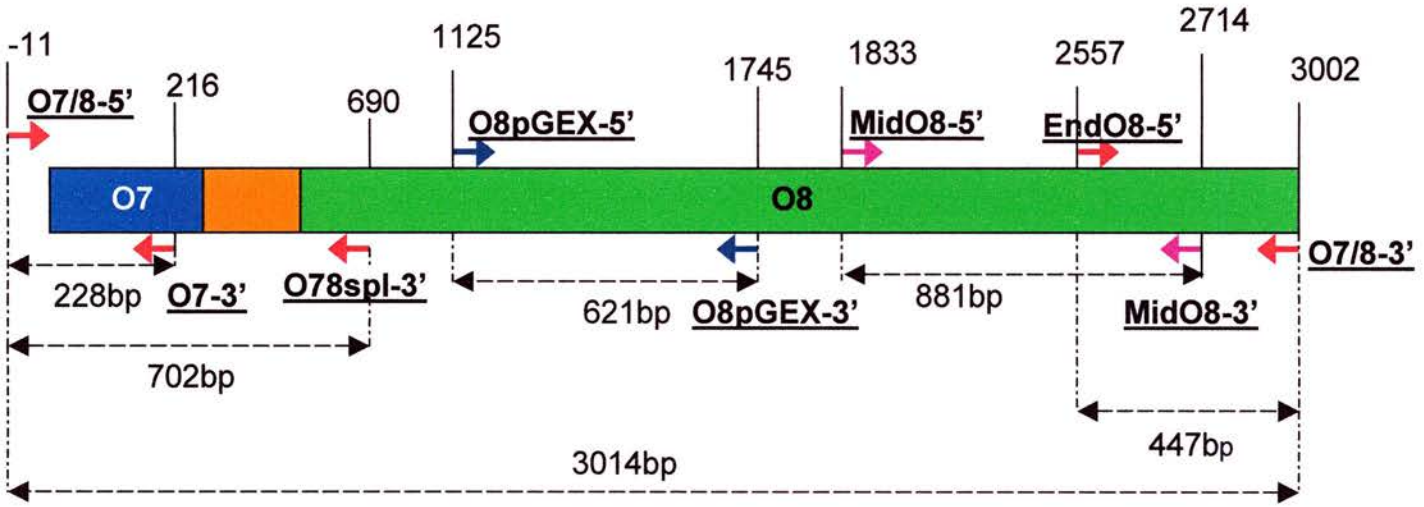


Figure 3.3.1.10 Possible antigenic sites of the translated O7/O8 sequence were predicted using the ANTIGENIC program. The translated O7/O8 sequence is shown with numbers shown above the amino acids for orientation. The O7 sequence is shown with a blue dashed overline and the remaining sequence is O8 sequence. Antigenic sites are underlined in red, with the maximum score amino acid also shown in red. Sequence used in generation of the anti-O8 antibody is shown in green.

An aliquot of 2 x 10^6 BJ/880 OvHV-2 infected rabbit cells was incubated with 3mM sodium butyrate overnight at 37°C. Total RNA was then extracted from these cells and from untreated BJ/880 cells using a QIAGEN RNeasy kit as described (Section 2.7.2). The RNA was quantified by spectrometry and 5μg RNA from two samples of BJ/880 cells, 1 and 2 and from the sodium butyrate treated BJ/880 cells, was analysed by northern blotting as described (Section 2.7.4). The blot was probed using a ^{32}P labelled OvHV-2 O8 probe. This was synthesised by PCR amplification of OvHV-2 DNA using the primers O8pGEX-5' and O8pGEX-3' (Appendix 2). These primers amplified a 621bp region of OvHV-2 O8 (Figure 3.3.2.1). The membrane was exposed to x-ray film overnight, however no signal was detected. The membrane was then exposed to film for a further 2 weeks, however no signal was detected. In order to check for the presence of RNA on the membrane, it was re-probed using a ^{32}P labelled probe for the housekeeping gene glyceradehyde-3-phosphate dehydrogense (GAPDH). This was made by PCR amplification of a 400bp region of the rabbit GAPDH gene using the PCR primers Rgap-5' and Rgap-3' (Appendix 2). Exposure of the membrane to film overnight showed the presence of GAPDH mRNA at 1.5kb (Figure 3.3.2.2). It was concluded that the lack of signal on the northern blots for OvHV-2 O8 was due to low expression of the mRNA rather than to lack of mRNA on the membrane. This experiment therefore showed that levels of O8 transcripts in the cell line were low and treatment of the cells with sodium butyrate did not increase expression of O8 to the level of detection.

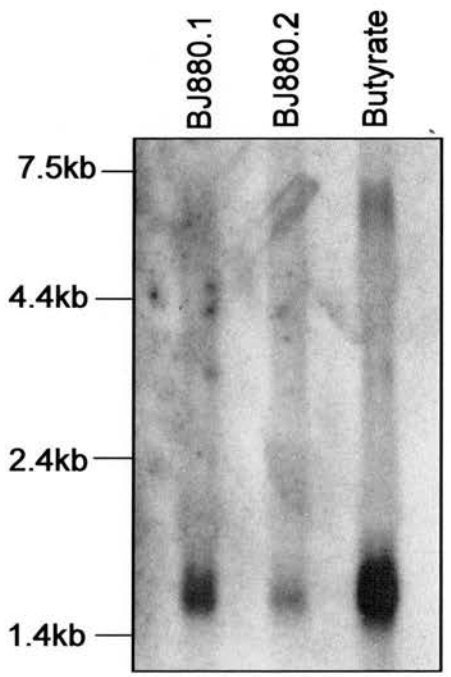
In an effort to increase sensitivity of the detection of O8 expression, poly A RNA was extracted from the OvHV-2 infected rabbit cell line BJ/2476 using an Oligotex Direct mRNA purification kit (Section 2.7.3) and quantified by spectrometry. RNA (10μg) was analysed by northern blotting as described (Section 2.7.4). Total RNA (10μg) extracted from MDBK cells, which had been previously transfected with an O7/O8 expression construct and had been shown by RT-PCR to express O8 (Section 3.3.3.7), was used as a positive control. The blot was probed using the ^{32}P labelled O8 probe. After exposure of the membrane to film overnight, a band corresponding to an RNA species of 2.2kb was detected in the transfected MDBK RNA lane (Figure 3.3.2.3), however no expression of OvHV-2 O8 RNA was detected after exposure for 2 further weeks. It was concluded therefore that levels of

Figure 3.3.2.1 Primers Used for PCR Amplification of the O7/O8 Region



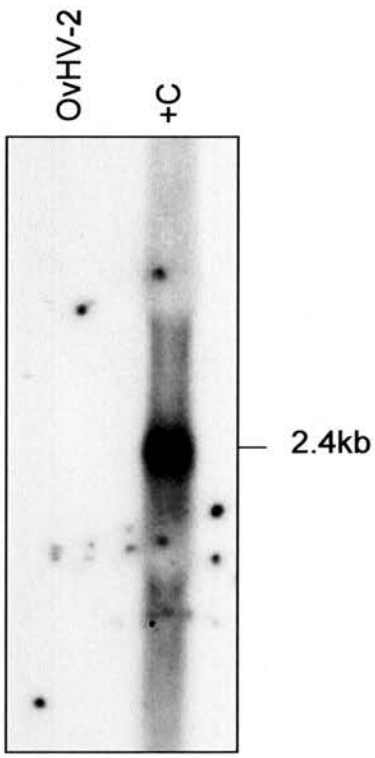
The diagram shows the O7/O8 region and the locations of primers used to amplify the sequence. The predicted O7 open reading frame is shown as a blue block, whilst the O8 open reading frame is shown in green. The area between the predicted open reading frames is shown in orange. Primer names are shown underlined and primer pairs are shown in the same colour. The coordinate number of the 5' end of the primer is shown with respect to the O7 translational start site, as is the length of the expected PCR product from amplification of unspliced DNA.

Figure 3.3.2.2 Northern Analysis of RNA Extracted From OvHV-2 Infected Rabbit Cells



RNA (5µg per sample) extracted from samples of the OvHV-2 infected rabbit cell line BJ/880 (1 & 2), and from BJ/880 cells incubated overnight with sodium butyrate, was analysed by northern blotting using an OvHV-2 O8 probe. No visible bands were observed after a 2 week exposure of the blot to film, therefore the blot was re-probed using a rabbit GAPDH probe. The membrane was exposed to x-ray film overnight. The size of the RNA species was determined by comparison with an RNA ladder.

Figure 3.3.2.3 Northern Analysis of Poly A RNA Extracted From the OvHV-2 Infected Rabbit Cell Line BJ/2476



Poly A RNA (10μg) was extracted from the OvHV-2 infected rabbit cell line BJ/2476 and analysed by northern blotting using an OvHV-2 O8 probe. The positive control (+C) was RNA extracted from MDBK cells transfected with an O7/O8 expression construct (section 3.3.3.6). The size of any bands was determined by comparison with a 1kb+ DNA ladder.

O8 transcripts in OvHV-2 infected rabbit cell lines were low and were below the level of detection that could be achieved by northern analysis of either total RNA or poly A RNA.

3.3.2.2 RT-PCR Analysis of The Expression of O7/O8 In OvHV-2 Infected Rabbit Cell Lines

Due to low levels of expression of O7/O8 as demonstrated by northern analysis, the expression of O7/O8 was analysed by RT-PCR as this technique is more sensitive than northern analysis. The O7/O8 transcript was predicted by sequence analysis to contain both the O7 and O8 open reading frames. The aim was to amplify the predicted cDNA transcript of O7/O8 by PCR and to clone this PCR product into a suitable vector for sequencing, so that the RNA structure and possible splice variants could be examined. PCR primers were therefore designed to amplify the O7/O8 transcript. The 5' end of the primer O7/8-5' was located at – 11 with respect to the translational start site of O7 and the primer contained a *Bam*HI restriction site at the 5' end (Appendix 2). The 5' end of the primer O7/8-3' was located at +3003 with respect to the O7 translational start site and the primer contained an *Eco*RI restriction site at the 5' end (Appendix 2). The primers were designed to amplify a 3kb product from genomic DNA that spanned both the O7 and O8 open reading frames (Figure 3.3.2.1). The restriction sites were designed to facilitate cloning of any RT-PCR products into the vector pBluescript KS for sequencing.

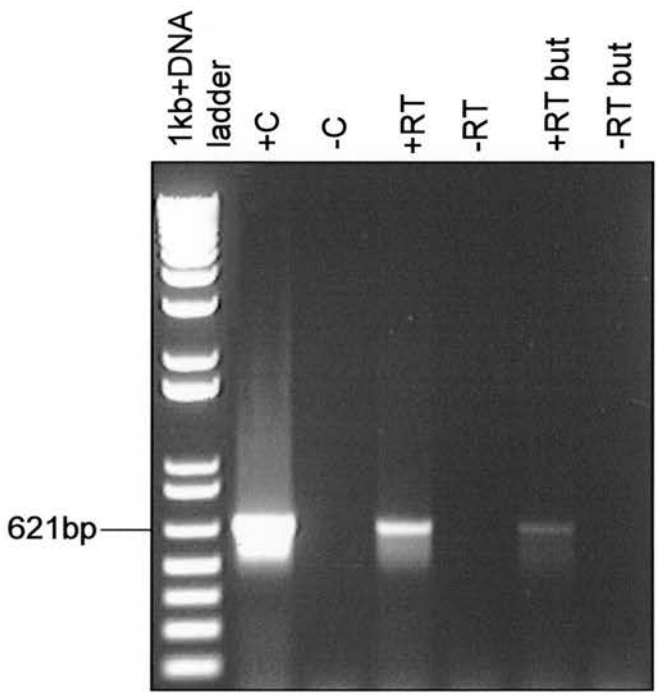
Total RNA (1μg of each sample) extracted from the sodium butyrate treated BJ/880 rabbit cell line and from non-butyrate treated BJ/880 cells was reverse transcribed into cDNA using superscript II RT enzyme (Section 2.7.5) and random primers. For each RT reaction a negative control reaction was included, which contained 1μg RNA but no reverse transcriptase (RT) enzyme. This was to check that any PCR product from the RT reactions was produced by amplification of cDNA and not from amplification of contaminating DNA in the RNA samples. The BJ/880 cDNA was used as a template for PCR amplification, initially using the GAPDH primers Rgap-5' and Rgap-3' (Appendix 2), in order to establish that the reverse transcription reaction had worked. In order to check that O8 was expressed in the BJ/880 cell line PCR amplification of BJ/880 cDNA was performed using the

primers O8pGEX-5' and O8pGEX-3' (Appendix 2), which amplify a 621bp section of O8 (Figure 3.3.2.1). It was possible to amplify the 621bp section of O8 in all cDNA samples (Figure 3.3.2.4). This demonstrated that there was expression of OvHV-2 viral genes in the samples, therefore PCR amplification of the O7/O8 cDNA product was attempted.

The aim was for resulting PCR products to be cloned and sequenced; therefore the most accurate PCR amplification possible was required. The PCR reaction was initially optimised with BJ/1035 genomic DNA as a template, using the proof-reading DNA polymerase *pfu* turbo (Stratagene). Extension times from 3 - 4 minutes were tested, and annealing temperatures from 50°C to 53°C, using 40 cycles of PCR, however the PCR reaction proved unreliable and difficult to optimise. Optimisation was repeated using *Taq* DNA polymerase, as this enzyme often produces more consistent amplification. This polymerase however, does not have proof-reading properties, therefore any products would have to be re-amplified and re-sequenced to confirm that the results were accurate. The optimal conditions for PCR amplification of the 3kb product using *Taq* DNA polymerase were an annealing temperature of 52°C and an extension time of 3 minutes 30 seconds. After optimisation, the same conditions were used for PCR amplification of the BJ/880 cDNA, however no product was produced. It was thought that the possible reason for the lack of O7/O8 PCR product were that possibly the full length cDNA template was not being produced using the random primers. It is also known that longer products amplify less efficiently than shorter products. The RT reaction was repeated using superscript II RT enzyme and oligo dT primers, in order to improve the yield of cDNAs containing the 3' end of the sequence. PCR amplification was again performed using the O7/8-5' and O7/8-3' primers, however no product was seen.

Due to the difficulty in obtaining the O7/O8 RT-PCR product from BJ/880 RNA, it was decided to study the transcription pattern of O7/O8 by RT-PCR amplification of selected smaller areas of the sequence. In order to do this, PCR primers were designed to amplify selected areas of O7 and O8, some of which spanned predicted splice sites. The primer pairs used are shown on Figure 3.3.2.1. PCR amplification of BJ/880 cDNA was performed using the primer pair O7/8-5' and O7-3' to show O7 was expressed. The primer pair O7/8-5' and O7/8spl were

Figure 3.3.2.4 RT-PCR Analysis of RNA Extracted From OvHV-2 Infected Cell Line BJ/880 Using Primers to Amplify O8



Total RNA was extracted from the OvHV-2 infected cell line BJ/880 and from BJ/880 cells after a 24 hour incubation with sodium butyrate (but). The RNA was reverse transcribed using random primers and the products used as a template for PCR amplification using the primers O8pGEX-5' and O8pGEX-3'. The PCR products were then analysed by agarose gel electrophoresis alongside a 1kb+ DNA ladder.

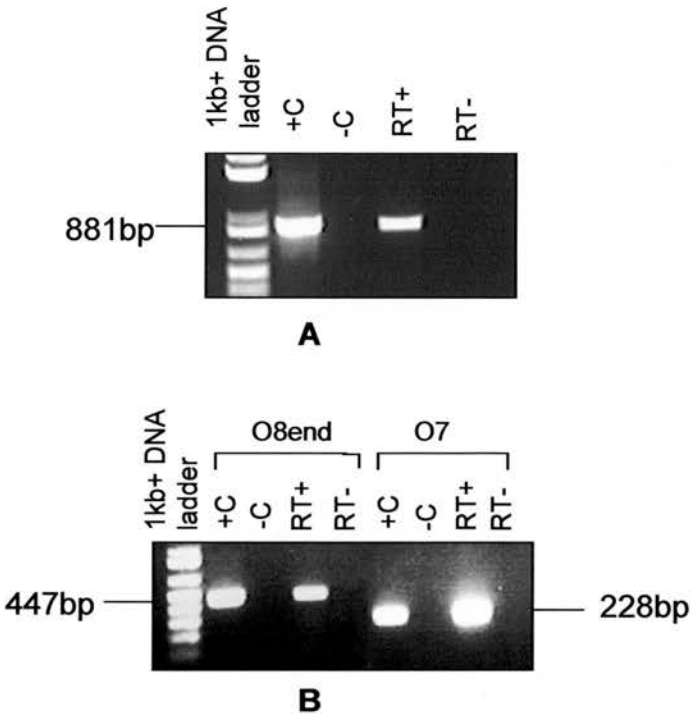
+C = PCR Positive control using OvHV-2 genomic DNA template. –C = PCR negative control using dH_2O template. RT+ = RT-PCR product. RT– = RT-PCR control omitting reverse transcriptase enzyme.

designed to amplify sequence across the region from O7 to O8, in order to show that a cDNA encoding both O7 and O8 was produced. O8mid-5' and O8mid-3' were designed to amplify the middle region of O8. O8end-5' and O7/8-3' spanned the putative splice site across from the O8a ORF to O8b ORF, therefore PCR amplification using these primers would confirm if this splice occurs. PCR amplification was performed using these primer pairs with BJ/880 cDNA as template and OvHV-2 DNA as positive control template. Product was identified from all the PCR reactions; therefore this showed that there was expression of the O7/O8 region in the areas of O7/O8 tested (Figure 3.3.2.5).

The primers O7/8-5' and O7/8 spl produced a 702bp product when used for PCR amplification of genomic DNA (Figure 3.3.2.6). The O7 and O8 open reading frames are predicted to be spliced together to produce the O7/O8 glycoprotein mRNA, therefore a smaller PCR product than that produced by amplification of the control genomic DNA was expected. Only small amount of product was generated in the RT-PCR and was not easily visualised on examination of the agarose gel under UV light. The product appeared the same size as that produced by PCR amplification of genomic DNA.

In order to determine if the PCR product was O7/O8 DNA the RT-PCR product was analysed by Southern blotting. The RT-PCR product produced from PCR amplification of BJ/880 cDNA with the primers O7/8-5' and O7/8 spl was electrophoresed and the DNA transferred to Magnagraph nylon transfer membrane (Section 2.2). The membrane was then probed using ^{32}P labelled cosmid C57, as this cosmid contained the O7/O8 sequence. After exposure to x-ray film overnight, bands were clearly visible in both the positive control and the RT lane with no product in the negative control lanes (Figure 3.3.2.6). The positive control lane showed a number of spurious bands as well as the required product at 702bp. Two bands were also present in the RT lane; one at 700bp approximately and one seen at approximately 480 bp. Both the bands observed in the RT+ lane were present in the positive control lane, therefore it was concluded that there was no evidence of splicing occurring between the O7 and O8 open reading frames. The fact that a product resulted from this amplification reaction was evidence that O7 and O8 were

Figure 3.3.2.5 RT-PCR Analysis of RNA Extracted From the OvHV-2 Infected Cell Line BJ/880



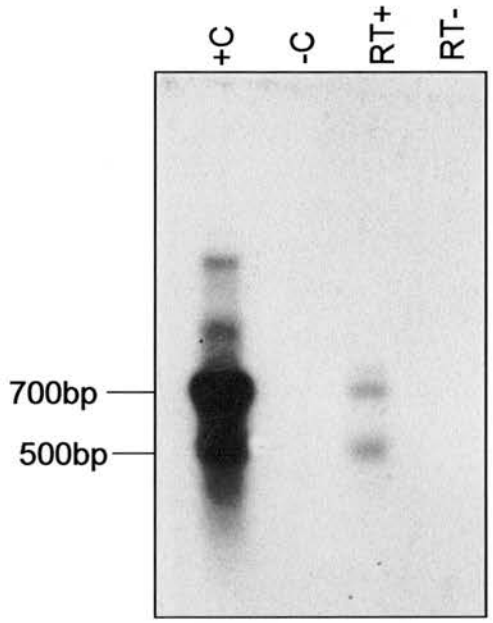
Total RNA was extracted from OvHV-2 infected cells and reverse transcribed using random primers. The RT product was used as a template for PCR amplification. The PCR products were analysed by agarose gel electrophoresis alongside a 1kb+ DNA ladder. The sizes of the products are indicated.

A – Products of PCR amplification of the cDNA using primers O8mid-5' and O8mid-3' which amplify the middle region of O8.

B – Products of PCR amplification of cDNA using primers O7/8-3' and O8end-5' which amplify sequence at the 3' end of O8 (labelled O8end) or the primers O7/8-5' and O7-3' which amplify O7 sequence (labelled O7).

+C = PCR Positive control using OvHV-2 genomic DNA template. –C = PCR negative control using dH_2O template. RT+ = RT-PCR product. RT– = RT-PCR control omitting reverse transcriptase enzyme.

Figure 3.3.2.6 Southern Analysis of the Products From RT-PCR Analysis of RNA Extracted From the OvHV-2 Infected Cell Line BJ/880



Total RNA was extracted from the OvHV-2 infected cell line BJ/880 and reverse transcribed using random primers. The product was then used for PCR amplification using the primers O7/8-5' and O7/8spl, which amplify sequence extending from O7 to O8. The PCR products were electrophoresed on a 1% agarose gel and analysed by Southern blotting using a cosmid C57 probe. The blot was exposed to x-ray film overnight and the sizes of the bands determined by comparison with a 1kb+ DNA ladder.

+C = PCR Positive control using OvHV-2 genomic DNA template. –C = PCR negative control using dH_2O template. RT+ = RT-PCR product. RT– = RT-PCR control omitting reverse transcriptase enzyme.

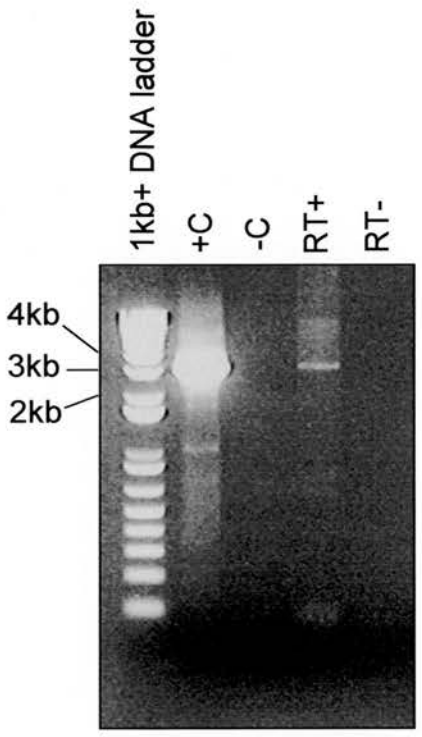
expressed in the same transcript. In order to confirm that the RT-PCR products were unspliced O7 and O8, it would have been necessary to clone the RT-PCR products into a suitable vector for sequencing, however this was not carried out due to the small amount of PCR product present.

3.3.2.3 RT-PCR Analysis of Poly A RNA Extracted From OvHV-2 Infected Rabbit Cell Lines

Until this point, RT-PCR analysis had only been carried out using total RNA. RT-PCR analysis of poly A RNA would increase the chances of detection of viral transcripts, however this had not been carried out due to insufficient numbers of OvHV-2 infected cells for analysis. The availability of a new OvHV-2 infected cell line named BJ/2476, meant that extraction of poly A RNA was now possible. Poly A RNA was extracted from the OvHV-2 infected rabbit cell line BJ/2476 using an oligotex direct mRNA kit as described (Section 2.7.3). Poly A RNA (1μg) was the reverse transcribed using superscript II RT enzyme and oligo dT primers. A sample of RNA was prepared for the reverse transcription reaction omitting the reverse transcriptase enzyme, as a negative control (RT-) to ensure that any amplification from the samples was not due to amplification of contaminating genomic DNA. The reverse transcribed samples, both with RT enzyme and without RT enzyme, were used as templates for PCR amplification.

Initially PCR amplification was performed using the GAPDH primers Rgap-5' and Rgap-3' (Appendix 2). This confirmed that the RT reactions had worked and PCR amplification was then performed using the O7/8-5' and O7/8-3' primers (Appendix 2), designed to amplify the full length O7/O8 cDNA product (Figure 3.3.2.1). The PCR enzyme Expand Long Template PCR system (Roche) was used. This was because the enzyme had proved very efficient for the amplification of relatively long templates when little template was present as was likely in this case. PCR amplification of the BJ/2476 cDNA resulted in a product approximately 3kb in length, which appeared the same size as that produced by PCR amplification of positive control genomic DNA (Figure 3.3.2.7). In order to sequence this RT-PCR product TA cloning of the PCR product was attempted, however this was not successful.

Figure 3.3.2.7 RT-PCR Analysis of Poly A RNA Extracted From the OvHV-2 Infected Rabbit Cell Line BJ/2476



Poly A RNA was extracted from the OvHV-2 infected rabbit cell line BJ/2476. RNA (1μg) was reverse transcribed using oligo dT primers. The RT product was amplified by PCR using the primers O7/8-5' and O7/8-3', which amplify the O7/O8 sequence. The PCR products were then analysed by agarose gel electrophoresis alongside a 1kb+ DNA ladder.

+C = PCR Positive control using OvHV-2 genomic DNA template. –C = PCR negative control using dH_2O template. RT+ = RT-PCR product. RT– = RT-PCR control omitting reverse transcriptase enzyme.

The production of this full length O7/O8 PCR product by amplification of cDNA from the OvHV-2 rabbit cell line BJ/2476 provides further evidence that O7 and O8 are expressed on the same transcript, however there was no evidence of splicing of the transcript. In conclusion therefore, analysis of OvHV-2 infected rabbit cell lines has provided evidence that as predicted, the O7 and O8 open reading frames are expressed on the same transcript, in agreement with the prediction that they both form functional domains of a putative viral glycoprotein. There was no evidence that the OvHV-2 O7/O8 transcripts expressed in OvHV-2 infected rabbit cell lines are spliced.

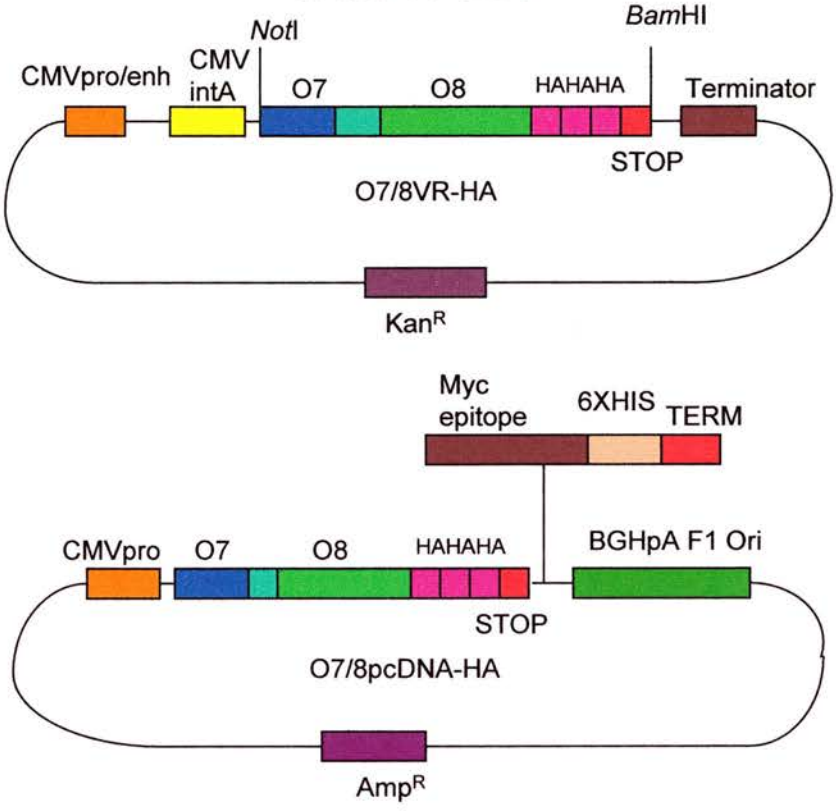
3.3.3 Analysis of O7/O8 Expression In Transfected Cells

3.3.3.1 Cloning O7 and O8 Into pVR1255

Analysis of the expression of O7/O8 in OvHV-2 infected cell lines was difficult due to the low levels of expression of viral genes in these cells and due to the short life of OvHV-2 infected cell lines restricting availability of cells for analysis. In order to further analyse the splicing and expression pattern of O7/O8 and to avoid the problem of low expression of these genes in virus infected cell lines, the region of the genome containing O7 and O8 was cloned into the expression vector pVR1255 in preparation for transfection into eukaryotic cells.

A 3' influenza haemagglutinin (HA) epitope tagged O7/O8 construct was made (Figure 3.3.3.1), as this would enable protein expression of the construct to be studied using commercially available anti-HA antibodies. The vector pVR1255 was obtained under licence from Vical Inc. (Hartikka *et al.*, 1996). This vector was chosen as it contained a CMV promoter sequence for high level of expression of cloned genes. The vector also contained the CMV intron A sequence. This sequence is thought to increase gene expression due to an enhanced rate of polyadenylation and nuclear transport associated with RNA splicing (Huang & Gorman, 1990). It is also thought that this element may be a positive element for the high efficiency of the CMV promoter/enhancer (Xu *et al.*, 2001). PCR primers were designed to amplify the O7/O8 sequence such that PCR amplification of OvHV-2 DNA template would

Figure 3.3.3.1 Expression Constructs Used for Transfection of the O7 and O8 ORFs



To make the constructs the region spanning the O7 and O8 open reading frames was amplified by PCR using the primers O7/8HA-5' and O7/8HA-3'. The PCR product was digested with *Not*I and *Bam*HI and ligated into the vector pVR1255 to make O7/8VR-HA, or into the vector pcDNA3.1 myc His (-) to make the construct O7/8pcDNA-HA. Each construct contained the predicted O7 and O8 open reading frames and the region between O7 and O8 followed by three haemagglutinin epitopes (YPYDVPDYA) and a stop codon. The construct O7/8VR-HA contained a CMV intron A sequence (CMV int A) downstream of the CMV promoter/enhancer sequence (CMV pro/enh). In the construct O7/8pcDNA-HA the O7/O8 sequence was driven by the CMV promoter only (CMVpro).

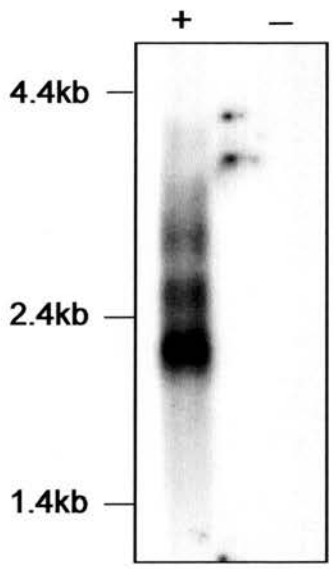
produce a haemagglutinin tagged O7/O8 construct, which could then be cloned into the vector PVR1255 using the *Not*I and *Bam*HI sites in this vector. The 5' end of PCR primer O7/8HA-5' was at –18 with respect to the O7 translation start site and contained a *Not*I site at the 5'end (Appendix 2). The 5' end of the primer O7/8HA-3' was located at the 3' end of the O8 open reading frame (+3002) and contained three copies of the haemagglutinin epitope sequence (YPYDVPDYA) followed by an ATG stop codon and a *Bam*HI restriction site (Appendix 2).

PCR amplification of BJ/1035 OvHV-2 DNA was performed using the primers O7/8HA-5' and O7/8HA-3' using *Taqplus Long* DNA polymerase enzyme (Stratagene). This PCR enzyme was designed for better results on amplification of long PCR products and also had proof-reading properties. The conditions that produced the PCR product of the required length of 3 kb were an extension time of 3 mins 30 s and an annealing temperature of 57°C. This was repeated for 30 cycles. The PCR product was termed O7/8-HA and was purified before restriction digestion with *Bam*HI and *Not*I. The digested PCR product was then ligated to *Bam*HI and *Not*I digested pVR1255. The products of the ligation were transfected into the XL-1 Blue strain of *E. coli* and transformants containing the construct were selected by restriction analysis of mini preparations of plasmid DNA from selected colonies. A positive clone containing an insert of the correct size was selected and named O7/8VR-HA. In order to prepare pure DNA for transfection, a large scale preparation of O7/8VR-HA was carried out using an endo-free maxi prep kit as described (Section 2.1.14).

3.3.3.2 Northern Analysis of RNA Extracted From Transfected HEK293 Cells

O7/8VR-HA DNA (20μg) was transfected into HEK293 cells by electroporation, using a double pulse protocol (Section 2.10.8). Negative control cells were also subjected to electroporation, but with no plasmid DNA (mock tranfections). After 48 hours the transfected cells were harvested and total RNA extracted from the cells using a QIAGEN RNeasy kit. The RNA extracted was used for northern analysis and the blot probed using the O8 probe. Exposure of the blot to film for 7 days produced

Figure 3.3.3.2 Northern Analysis of Poly A RNA Extracted From O7/O8 Transfected HEK293 Cells



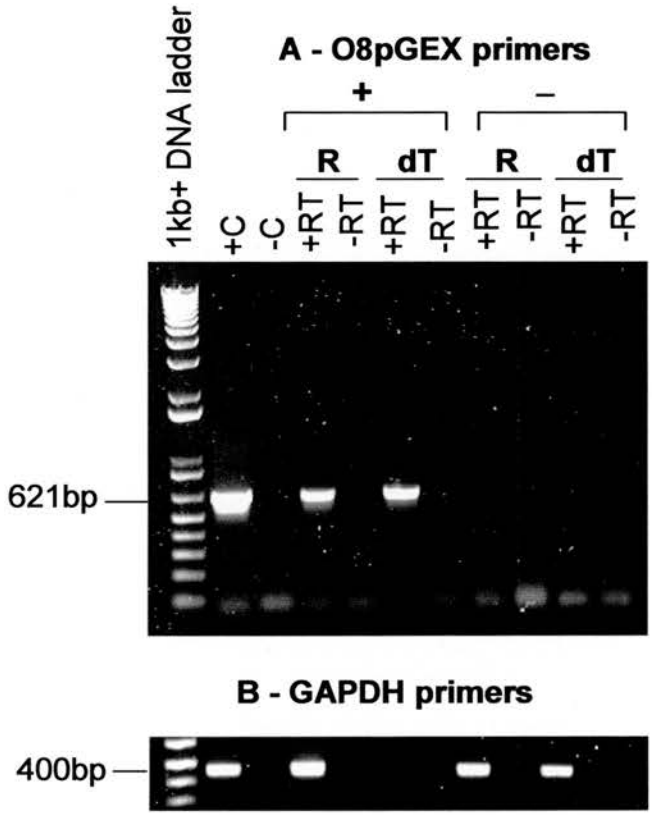
HEK293 cells were transfected with the construct O7/8VR-HA and the cells harvested after 48 hours. Poly A RNA was extracted from transfected (+) and untransfected (-) cells and analysed by northern blotting using an OvHV-2 O8 probe. The blot was exposed to x-ray film for 7 days and the sizes of the RNA bands determined by comparison with an RNA size marker.

3 bands representative of RNA species of approximately 2.2kb, 2.4kb and 3.0kb. Since the size of the genomic O7/O8 sequence was 3.0kb, this was evidence that the O7/O8 construct was being spliced in HEK293 cells. It was suspected that the three different sizes of RNA observed were due to different stages of splicing of immature transcripts. In order to test this, the transfection was repeated as before and poly A RNA extracted from the harvested cells using an oligotex direct mRNA kit (Section 2.7.3). The poly A RNA (2μg of each sample) was analysed by northern blotting and the blot probed using the O8 probe. Exposure of the blot to film for 7 days produced the same 3 RNA species seen on analysis of total RNA of approximately 2.2kb, 2.4kb and 3.0kb (Figure 3.3.3.2). The fact that bands representing three RNA species of the same size were identified on analysis of total RNA and poly A RNA suggested that all bands seen represented mature polyadenylated transcripts. The 3kb RNA species was the correct size to represent an unspliced O7/O8 transcript, and the smaller sized RNA species were evidence that the O7/O8 construct was spliced in HEK293 cells.

3.3.3.3 RT-PCR Analysis of RNA Extracted From O7/O8 Transfected HEK293 Cells

In order to analyse the sequence of the O7/O8 transcripts seen after transfection of HEK293 cells with the O7/8VR-HA plasmid, RNA extracted from the cells was analysed by RT-PCR and the PCR products cloned into the vector PKS for sequencing. Total RNA (1μg) extracted from O7/8VR-HA transfected 293 cells was reverse transcribed using Superscript II RT enzyme and oligo dT primers as described (Section 2.7.5). For all RNA samples a control reverse transcriptase reaction was set up omitting RT enzyme in order to show that any RT-PCR products resulted from amplification of cDNA and not from contaminating DNA in the RNA samples. The products of the RT reactions were used initially as a template for PCR amplification using the primers Hgap-5' and Hgap-3' (Appendix 2) (Obtained from Dr S.J. Talbot), which were designed to amplify the human GAPDH gene. A product of the correct size of 400bp was produced in all RT samples suggesting that the RT reactions had worked (Figure 3.3.3.3B). PCR amplification of the cDNA was then performed using the primers O8pGEX-5' and O8pGEX-3,' to check for expression

Figure 3.3.3.3 RT-PCR Analysis of RNA Extracted From O7/O8 Transfected HEK293 Cells using Primers to Amplify O8



HEK293 cells were transfected with the construct O7/8VR-HA. Total RNA was extracted from transfected (+) and untransfected (–) cells after 48 hours and used in reverse transcriptase reactions using random primers (**R**) or oligo dT primers (**dT**). The RT products were then amplified by PCR. **A** – Products of PCR amplification using the primers O8pGEX-5' and O8pGEX-3', which amplify O8. **B** – Products of PCR amplification using the primers Hgap-5' and Hgap-3' (obtained from Dr S.J. Talbot), which amplify a 400bp section of the human GAPDH gene. The PCR products were analysed by agarose gel electrophoresis alongside a 1kb+ DNA ladder.

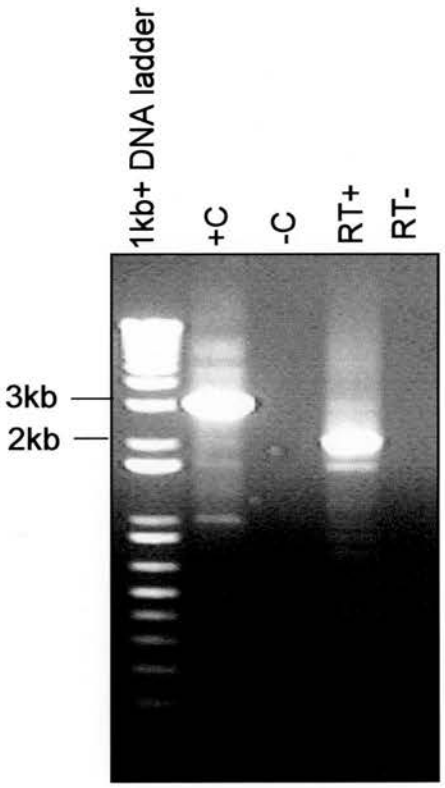
+C = PCR Positive control using OvHV-2 genomic DNA template with O8pGEX primers and MHV-68 cDNA template with GAPDH primers. –C = PCR negative control using dH_2O template. RT+ = RT-PCR product. RT– = RT-PCR control omitting reverse transcriptase enzyme.

of O8 in the transfected cells before attempting amplification of the full length O7/O8 product. A product of the correct size of 621bp was amplified from all the O7/O8 transfected cells, but not from the mock-transfected cells (Figure 3.3.3.3A), suggesting that the expression of O8 in the cells was as a result of the transfection with the O7/O8 construct. Finally amplification of the cDNA was performed using the using the O7/8-5' and O7/8-3' primers (Appendix 2). The PCR products were analysed by gel electrophoresis (Figure 3.3.3.4) and were seen to contain a major product of size approximately 2.2kb and minor products of various sizes. The PCR product was purified using the CONCERT PCR purification system (Invitrogen) and digested using *Bam*HI and *Eco*RI. The digested PCR product was then ligated to *Bam*HI and *Eco*RI digested pBluescribe KS II vector (Stratagene) (Appendix 5) and the ligated DNA transfected into the XL-1 Blue strain of *E. coli*. Colonies were selected and analysed for the presence of inserted DNA by restriction digestion and electrophoresis of plasmid DNA purified from bacterial colonies. Positive colonies with inserted DNA of various sizes were found and samples with inserted DNA of size approximately 2.2kb, 1.9kb and 1.6kb were sequenced.

Three different spliced cDNAs were found (Figure 3.3.3.5). O7/8-293.11 contained just one large splice of size 943bp in O8. The area spliced out extended from 1857 to 2800 with respect to the translational start of O7. The splice sites used were exactly as predicted by analysis using the NetGene2 program; the 5' splice donor site in O8 with a confidence value of 0.37 and the splice acceptor site at 2800 with a confidence level of 0.94 (confidence values are given relative to the cutoff values used to find nearly all true splice donor or acceptor sites). The area spliced out of the O8 corresponded closely with the area of O8 not homologous to A8 of AlHV-1. The lack of splicing in O7 meant that in transcribing from O7 to O8 several stop signals were found, therefore it was unlikely that an O7/O8 protein would be translated from this transcript.

O7/8-293.6 also contained a large splice from O8, however the splice donor site in O8 was at 1896 and the splice acceptor back into O8 was located at 2800 as before. The splice donor site at 1896 was predicted by NetGene, with a confidence value of 0.70 and produced an in frame O8. This cDNA also contained one small splice from within the predicted O7 ORF (from 42 -149) and one small splice in the

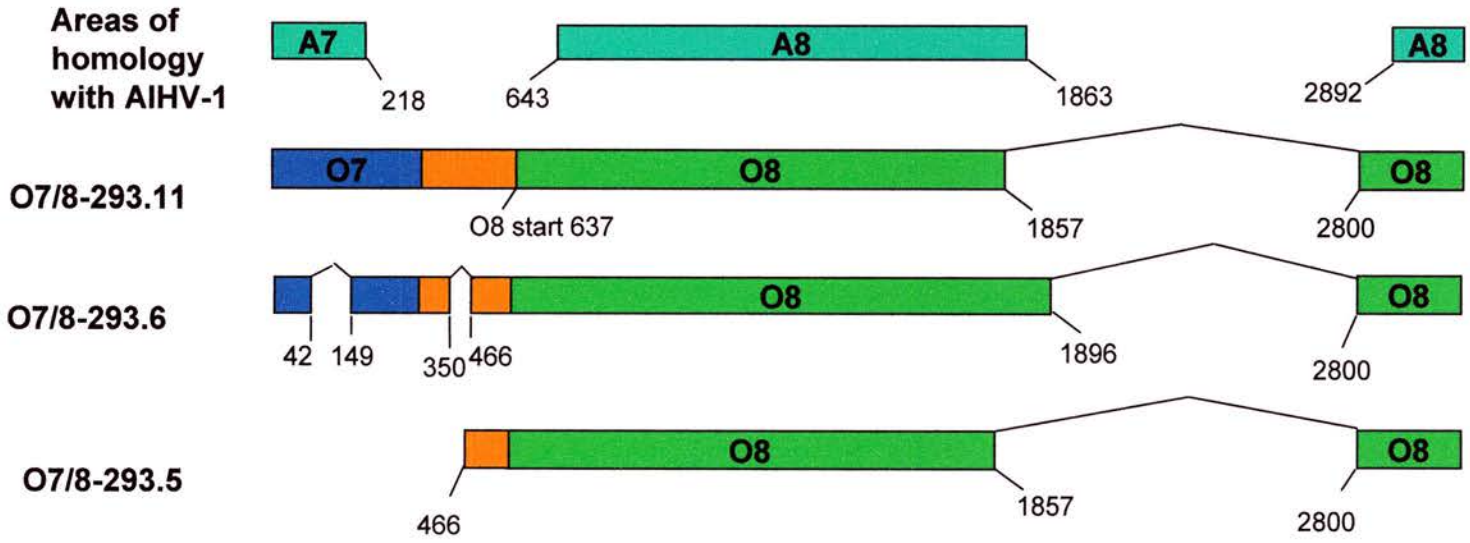
Figure 3.3.3.4 RT-PCR Analysis of RNA Extracted From O7/O8 Transfected HEK293 Cells Using Primers to Amplify O7/O8



HEK293 cells were transfected with the O7/8VR-HA construct and the cells harvested after 48 hours. Total RNA was extracted from the cells and used in reverse transcriptase reactions with oligo dT primers. The RT products were amplified by PCR using the primers O7/8-5' and O7/8-3'. The products were then analysed by agarose gel electrophoresis alongside a 1kb+ DNA ladder.

+C = PCR Positive control using OvHV-2 genomic DNA template. –C = PCR negative control using dH_2O template. RT+ = RT-PCR product. RT– = RT-PCR control omitting reverse transcriptase enzyme.

Figure 3.3.3.5 The Structure of cDNAs From O7/O8 Transfected HEK293 Cells



The O7/O8 construct O7/8VR-HA was transfected into HEK293 cells by electroporation and total RNA extracted from the cells after 48 hours. The RNA was subjected to RT-PCR analysis and the products cloned into the vector pBluescribe KS II and sequenced. The structure of sequenced PCR products is shown. The O7 open reading frame is shown in blue, and the O8 open reading frame is shown in green. The region between the open reading frames is shown in orange. The regions homologous to A7 and A8 of AlHV-1 are shown as turquoise blocks at the top of the page. The coordinates shown are nucleotide numbers relative to the start of the O7 open reading frame. The gaps in the O7/O8 sequence blocks represent regions where splicing has taken place.

region between O7 and O8 (350 - 466). The splice donor site at 42 was not predicted by analysis using the NetGene program, however the splice acceptor site was a consensus GT motif. The acceptor site at 149 was predicted with a confidence level of 0.96. The donor site at 350 was predicted with a confidence level of 0.55 and the acceptor site at 466 with a confidence level of 0.72. The splice in the O7 ORF meant that stop codons were introduced into the sequence, also following the second splice the sequence was not in frame with the predicted O8 ORF. This RNA was therefore unlikely to be translated into an O7/O8 protein. O7/8-293.5 extended from 466, a location 5' to the beginning of the O8 open reading frame, to the end of O8. The sequence contained the large 943bp splice in O8, using the same splice donor and acceptor sites that were used in O7/8-293.11. This PCR product was possibly generated by mis-priming of the O7/8-5' primer.

3.3.3.4 Cloning of O7 and O8 Into pcDNA 3.1/*Myc*-His (-)

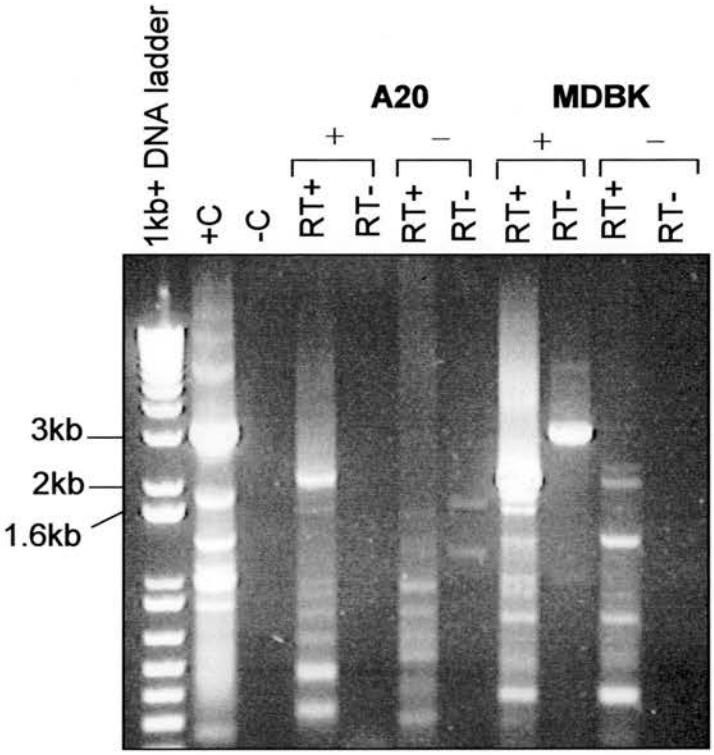
The plasmid pVR1255 contained the CMV intron A sequence (Figure 3.3.3.1), which was designed to improve expression of the inserted sequence. It was suspected that this sequence could potentially interfere with efficient splicing of the O7/O8 construct, therefore the O7/8-HA tagged construct was cloned into pcDNA3.1/*myc*-His (-) (Invitrogen) (Appendix 5). This vector was designed for high expression of inserted DNA under the control of the CMV promoter and since the construct contained a stop signal 3' to the triple HA tag, the *Myc*-His tag in the vector would not be expressed. Vector DNA was digested with *Not*I and *Bam*HI before desphosphorylating using the CIAP enzyme. The pcDNA vector was then ligated to *Not*I and *Bam*HI digested O7/8-HA DNA and the construct transfected into the XL-1 Blue strain of *E. coli*. Colonies were selected and analysed for the presence of plasmid DNA containing an insert of the correct size of 3kb, by digestion of minipreparations of plasmid DNA with *Bam*HI and *Not*I. A positive colony was selected and a large-scale preparation of plasmid DNA prepared using a QIAGEN Endo Free Plasmid Maxi Kit. This O7/O8 construct was named O7/8pcDNA-HA (Figure 3.3.3.1).

3.3.3.5 Transfection of The O7/O8 Sequence Into A20 Cells

The splicing patterns found in the cDNAs from transfection of HEK293 cells with the O7/O8 construct were such that the open reading frames were not maintained in any of the cDNAs. It was thought that this was possibly because cell type or species specific factors were involved in splicing, or that correct splicing required viral factors. Since OvHV-2 is suspected to infect lymphocytes in cattle and sheep (Baxter *et al.*, 1993), it was possible that lymphocyte specific factors were involved in splicing. In order to address this, the O7/8pcDNA-HA construct (10μg) was transfected into A20 cells (Kim *et al.*, 1979). These cells are a mouse B cell lymphoma line and were known to transfect efficiently. The A20 cells were transfected using a double pulse protocol as described (Section 2.10.8) and 20μg DNA was used for each transfection. A control plasmid containing the green fluorescent protein (GFP) gene (pEGFPC1, Clontech), was transfected to assess transfection efficiency. The optimum efficiency obtained in these cells was approximately 50% and the electroporation protocol used resulted in a high percentage of cell death.

After 48 hours the cells were harvested and total RNA extracted from the cells using a QIAGEN RNeasy kit as described previously (Section 2.7.2). The RNA was quantified by fluorometry. The yield of RNA from A20 cells was low, possibly because of the high percentage of cell death resulting from electroporation. The RNA was analysed by RT-PCR in the same way as for the HEK293 cells using the O7/8-5' and O7/8-3' primers. The PCR products were then analysed by gel electrophoresis. The major product of the RT-PCRs was approximately 2.2kb in length (Figure 3.3.3.6). This was the same size of RT-PCR product as that resulting from the same experiment in HEK293 cells. RNA extracted from A20 cells was not analysed by northern blotting, as the amount of RNA extracted from the cells was not sufficient for this method. It was concluded therefore, that there were no differences in the splicing pattern of the O7/O8 construct in A20 cells as compared to those found in HEK293 cells. This suggested that B lymphocyte cell specific factors were not required for splicing of the O7/O8 construct.

Figure 3.3.3.6 RT-PCR Analysis of RNA Extracted From O7/O8 Transfected A20 and MDBK Cells



A20 cells and MDBK cells were transfected with the construct O7/8pcDNA-HA by electroporation and total RNA extracted after 48 hours. RNA from transfected (+) and non-transfected (-) cells was used in reverse transcription reactions with oligo dT primers. PCR amplification of the RT products was performed using the primers O7/8-5' and O7/8-3' which amplify O7/O8 sequence. The PCR products were analysed by electrophoresis on a 1% agarose gel and are shown compared to a 1kb+ DNA ladder.

+C = PCR Positive control using OvHV-2 genomic DNA template. –C = PCR negative control using dH_2O template. RT+ = RT-PCR product. RT– = RT-PCR control omitting reverse transcriptase enzyme.

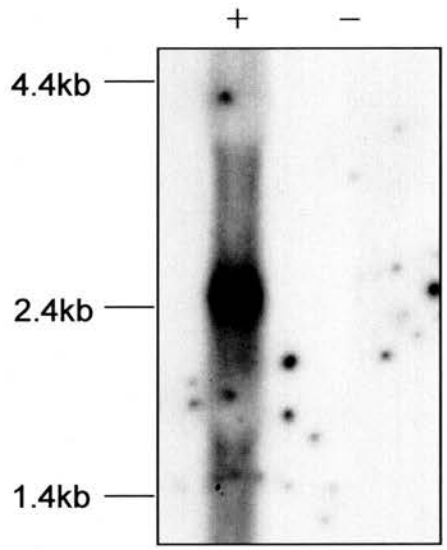
3.3.3.6 Northern Analysis of Transfected MDBK Cells

To determine if splicing was affected by species-specific factors the O7/8pcDNA-HA construct was transfected into Madin-Darby Bovine Kidney (MDBK) cells (Madin, 1958) obtained from the Moredun Research Institute. This cell type was chosen because OvHV-2 naturally infects cattle and MDBK cells were known to transfect efficiently. Transfection efficiency in MDBK cells by electroporation was optimised, by testing a number of different single and double pulse protocols using the GFP expressing plasmid pEGFPC1. A single pulse protocol as described (Section 2.10.8) was found to give the optimal efficiency of approximately 70%. MDBK cells were transfected with the O7/8pcDNA-HA construct (10μg) using the single pulse electroporation protocol. Transfected cells were harvested after 48 hours and total RNA extracted using a QIAGEN RNeasy kit. RNA extracted from O7/8pcDNA-HA transfected MDBK cells was subjected to northern analysis using the O8 probe. This resulted in just one band at approximately 2.4kb after a two day exposure of the membrane to film (Figure 3.3.3.7).

3.3.3.7 RT-PCR Analysis of Transfected MDBK Cells

In order to analyse the sequence of O7/O8 RNAs extracted from transfected MDBK cells, the RNA was analysed by RT-PCR using the O7/8-5' and O7/8-3' primers. Gel electrophoresis of the RT-PCR products showed that the same main product of 2.2kb was found in O7/8pcDNA-HA transfected MDBK cells (Figure 3.3.3.6). A product of size 3kb was found in the RT- control indicating that there was some O7/8pcDNA-HA plasmid DNA contamination in the RNA sample. The 2.2kb RT-PCR product from RT-PCR analysis of RNA extracted from transfected MDBK cells and associated products close to it, was excised from an agarose gel and purified using a QIAGEN Gel extraction kit as described. The PCR product was then ligated to the vector pKS using the *Bam*HI and *Eco*RI sites and the products of the ligation transfected into *E. coli*. A selection of 24 possible transformants was checked and all colonies proved negative for the presence of plasmids containing inserted DNA. The failure to clone the PCR products into pKS may have been due inefficient ligation, since the number of colonies obtained in the negative control ligations (plasmid without insert), was similar to the number obtained from the

Figure 3.3.3.7 Northern Analysis of RNA Extracted From O7/O8 Transfected MDBK Cells

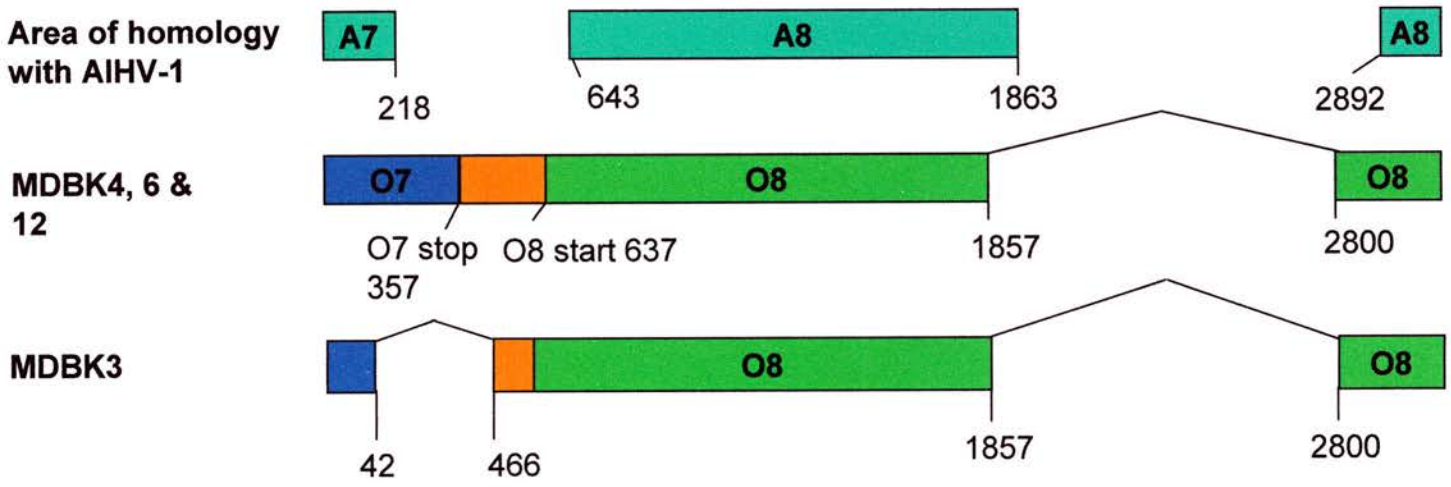


MDBK cells were transfected by electroporation with the construct O7/8pcDNA-HA. Transfected (+) and untransfected (-) cells were harvested after 48 hours and total RNA extracted. The RNA was analysed by northern blotting using an OvHV-2 O8 specific probe. The hybridised blot was exposed to film overnight, and the size of any RNA bands determined by comparison to an RNA size marker.

positive samples. The inefficient ligation reaction might have been due to the fact that restriction sites located at the end of DNA fragments such as PCR products sometimes digest poorly. The lack of properly digested PCR product as a substrate for ligation would result in a lower than optimum amount of ligated DNA. In order to solve this problem, the PCR amplification of the RNA extracted from transfected MDBK cells was repeated and the product excised from the gel and purified as before. The product was then ligated to the TA vector pGEM-T Easy (Promega)(Appendix 5) using the quick-ligation buffer supplied, as described (Section 2.4.12). The ligation reaction was then used to transform the XL-1 Blue strain of *E. coli.* Positive colonies were then identified by blue/white selection (Section 2.4.11). Plasmid DNA purified from positive colonies was analysed by restriction digestion with *Eco*RI, however none of the colonies analysed contained the correct inserted DNA and the plasmid DNA purified from these colonies did not digest with *Eco*RI.

Due to the unsatisfactory progress made by cloning using the pGEM-T Easy TA cloning vector, the O7/O8 PCR amplification was repeated and the product was cloned using a pCR 2.1 TOPO TA cloning kit, as this method had been successful in the past. The TOPO cloning reaction was set up as described (Section 2.4.10) and the products transfected into One Shot TOP10 competent *E. coli.* Positive colonies were again identified by blue/white selection and plasmid DNA purified from the colonies analysed by restriction enzyme digestion with *Eco*RI. Gel electrophoresis of the *Eco*RI digests showed that there were three samples with inserted DNA approximately 2.2kb in size (MDBK4, 6 and 12) and one sample with inserted DNA of size just over 1.6kb (MDBK3). These samples were sequenced and two different cDNA clones were observed (Figure 3.3.3.8). MDBK4, 6 and 12 were the same sequence; all contained a large splice within the O8 ORF at the same coordinates as seen in cDNAs from transfected HEK293 cells (1857 - 2800). MDBK3 contained a large splice in the O8 ORF from 1857 – 2800 in the same way as in MDBK 4, 6 and 12 and also a large splice from O7 from 42 – 466. The splice donor located at 42 was within the O7 ORF, whilst the splice acceptor at 466 was located in the region between the predicted O7 and O8 open reading frames. The splice acceptor at 466 meant that the following sequence contained stop signals and was not in the same

Figure 3.3.3.8 The Structure of cDNAs From O7/O8 Transfected MDBK Cells



The O7/O8 construct O7/8pcDNA-HA was transfected into MDBK cells by electroporation. Total RNA was extracted from the cells after 48 hours and the RNA used in RT-PCR analysis. The PCR products were cloned into the TA cloning vector pCR2.1 and sequenced. The structure of the sequenced PCR products is shown. The O7 ORF is shown in blue and the O8 ORF in green. The region between these two ORFs is shown in orange. The regions with homology to AlHV-1 are shown as turquoise blocks at the top of the page. The nucleotide coordinates are shown with respect to the start of the O7 open reading frame. The gaps in the O7/O8 sequence blocks represent regions where splicing has taken place.

frame as the O8 ORF. This RNA was therefore unlikely to be translated to a complete O7/O8 protein.

3.3.3.8 Western Blotting Analysis of O7/O8 Transfected HEK293 Cells

Western blotting analysis of the O7/O8 transfected HEK293 cells was carried out in order to determine the apparent molecular weight of the putative O7/O8 protein. Due to the presence of glycosylation, the molecular weight was expected to be higher than that predicted. Since the O7/O8 expression construct contained three HA epitopes at the 3' end, it was possible to use an anti-HA antibody to detect the protein. Initially untransfected HEK293 cells and cells transfected with 10, 15 or 20μg of the O7/8VR-HA plasmid were resuspended in western sample buffer at a concentration of 10^6/100μl. A 10 and 20μl aliquot of each cell lysate was analysed by SDS-PAGE as described (Section 2.8.1). After electrophoresis, samples of each cell lysate were analysed by Coomassie staining to ensure that cell lysis had been adequate. Samples were also transferred to a PVDF membrane by western blotting (Section 2.8.3). The western blot was incubated with the rat monoclonal anti-HA antibody, followed by a secondary biotinylated anti-rat IgG and a tertiary streptavidin-AP conjugate as described in Section 2.8.4. Antibody binding was detected by incubation of the membrane with Sigma FAST BCIP/NBT solution. A number of protein bands were identified on the membrane after incubation with the detection reagent, however all bands were present on the untransfected cells; therefore it was suspected that these were caused by non-specific binding of the antibodies.

It was suggested that the use of ECL might be required in order to detect the HA epitope (Alastair MacRae, personal communication). The western blot was repeated and HEK293 cells transfected with the control HA tagged plasmid pSG5/JκHA were used as a positive control. This plasmid was a triple HA tagged version of the signalling protein Jκ, inserted into the pSG5 expression vector (Stratagene) (Appendix 5) (Zhao *et al.*, 1996) and was obtained from Dr Jeff Sample, St Jude Children's Research Hospital, Memphis. Streptavidin-POD was used as a tertiary antibody as described (Section 2.8.4) and the ECL kit was used to detect antibody binding. No strong bands could be visualised in ECL analysis, therefore

optimisation of the protocol in terms of the concentrations of the antibodies used was required. Due to the time constraints, this was not pursued.

3.3.3.9 Immunofluorescence Analysis of O7/O8 Transfected MDBK Cells

It was not known if transfection of MDBK cells resulted in protein expression from the O7/O8 construct. Although the cDNAs sequenced seemed to have sequence containing stop codons, it was not known if protein translation occurred from these or other undetected transcripts. In order to investigate this, O7/8pcDNA-HA transfected MDBK cells were analysed by immunofluorescence using an anti-HA antibody (Section 2.10.2). This was possible because the O7/O8 expression construct contained three HA epitopes at the 3' end of the O8 open reading frame. Expression of the haemagglutinin epitopes therefore depended on translation of the construct right to the 3' end.

Two positive control constructs were used for transfection, both containing three haemagglutinin epitopes at the 3' end. The plasmid pSG5/Jκ-HA was a triple HA tagged version of the signalling protein Jκ, inserted into the pSG5 expression vector (Stratagene) (Appendix 5) (Zhao *et al.*, 1996). Plasmid pSG5/IRF7-HA was the pSG5 vector containing the inserted triple haemagglutinin tagged interferon regulatory factor 7 (IRF7) (Zhang & Pagano, 1997). Both plasmids were obtained from Dr Jeff Sample, St Jude Children's Research Hospital, Memphis. MDBK cells were transfected with the HA tagged constructs (10μg of each) using the single pulse electroporation protocol as described, and harvested after 48 hours. Transfection efficiency was checked using the pEGFPC1 plasmid and found to be approximately 60 - 70%. Harvested cells were resuspended in PBS at 10^6/ml, and "cytospun" onto Twinfrost slides (VWR) as described (Section 2.10.1). The "cytospun" cells were fixed with either 100% acetone or 4% paraformaldehyde (Section 2.10.1). Paraformaldehyde fixed cells were permeablised by incubation in PBS containing 0.2% Triton-X 100 for 5 - 10 minutes.

The fixed cells were subjected to immunostaining using the rat anti-HA antibody as described (Section 2.10.2). Both the primary anti-HA antibody and the secondary goat anti-rat-Ig antibody were used at a concentration of 1/250. The immunostained cells were examined by UV microscopy, however no fluorescence

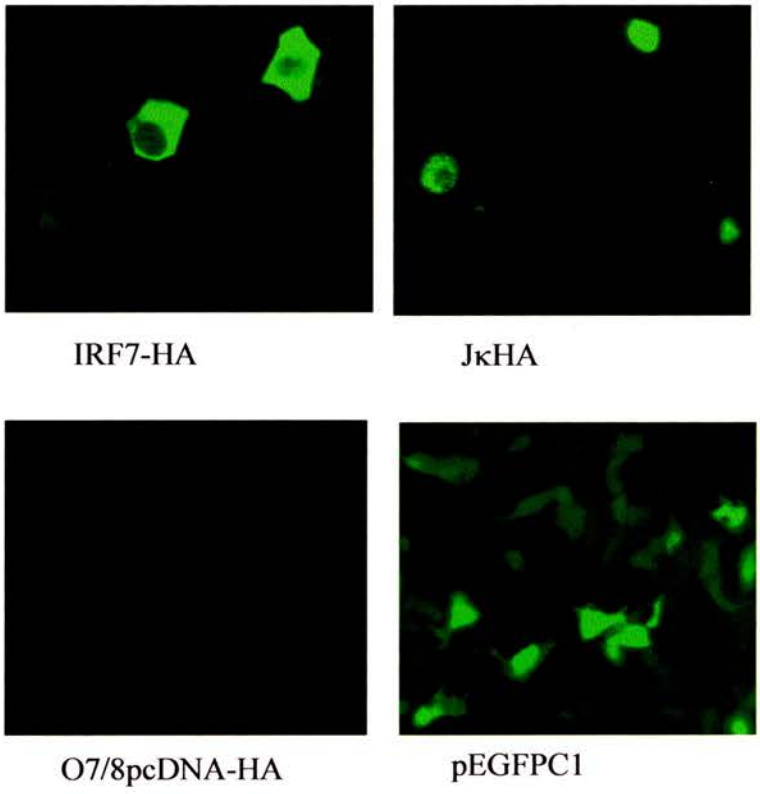
was observed in either the positive control samples or the O7/O8 transfected samples. The transfection efficiency using the GFP expressing control plasmid was high, however the same efficiency may not have been attained using the HA epitope tagged plasmids. Although the plasmid DNA was prepared using an endo-free maxi kit, it was still well known that higher transfection efficiencies could be obtained using DNA prepared using Caesium Chloride (CsCl) gradients. In order to ensure that the transfected DNA was of the highest quality possible, large scale preparations of the HA tagged plasmid DNA were prepared using CsCl gradients (Section 2.1.15). The transfections were repeated using the CsCl gradient prepared DNA samples and the cells cytospun and fixed as before. The paraformaldehyde fixed cells were permeablised for 5 minutes in 0.2% Triton-X 100 instead of 10 minutes as before, as overpermeablisation can destroy the protein epitopes used for antibody recognition. The fixed cells were immunostained using the rat anti-HA antibody in the same way as was done previously. On examination under the UV microscope, the positive control HA tagged proteins were visible in the paraformaldehyde fixed samples (Figure 3.3.3.9), but not in the 100% acetone fixed samples. The Jκ-HA protein localised in the cell nucleus and the IRF7-HA protein localised in the cytoplasm. No fluorescence was observed on the O7/8pcDNA-HA transfected cells, indicating that HA tagged proteins were not expressed from this construct in MDBK cells at levels detectable using the anti-HA antibody.

3.3.4 Production of An Antibody Against O8

Until this point, no evidence of expression of OvHV-2 proteins had been seen in OvHV-2 infected cell lines, therefore in order to detect the expression and subcellular location of the O8 protein in OvHV-2 infected cell lines, rabbits were immunised against a section of O8.

In order to raise an antibody to O8 a region of O8 was selected and amplified by PCR before cloning into the expression vector pGEX-3X. The vector pGEX-3X (Appendix 5) was constructed to direct the synthesis of foreign polypeptides in *E. coli* as fusion proteins with the C terminus of Sj26, a 26kDa glutathione S-transferase (GST) (Smith & Johnson, 1988). The plasmid directs the synthesis of

Figure 3.3.3.9 Immunofluorescence Analysis of MDBK Cells Transfected With Haemagglutinin Tagged Expression Constructs



The haemagglutinin tagged O7/O8 construct O7/8pcDNA-HA, the positive control haemagglutinin tagged constructs IRF7 and JκHA and the positive control plasmid pEGFPC1 were transfected into MDBK cells by electroporation. The pEGFP transfected cells were examined in the wells under UV light after 48 hours. The O7/8pcDNA, IRF7 and JκHA transfected cells were harvested after 48 hours and fixed to glass slides. The fixed cells were then subjected to immunostaining using a primary rat monoclonal anti-HA antibody followed by a secondary anti-rat FITC conjugate. The cells were then examined under UV light.

the fusion protein under the control of the IPTG inducible *tac* promoter. This is a strong promoter, which can result in high levels of expression of the fusion protein. The GST fusion protein is then purified by affinity chromatography on immobilised glutathione followed by competitive elution with excess reduced glutathione. On purification, the GST fusion protein can then be injected into rabbits in order to raise an antibody response.

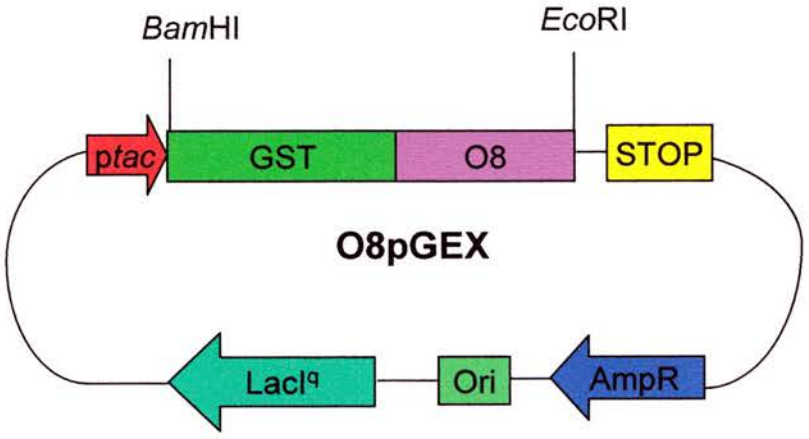
3.3.4.1 Expression of An O8-GST Fusion Protein

Primers were designed to amplify a 621bp (207 amino acids) (aa 292-549) region of O8 (Figure 3.3.1.9) between +1125 and +1746 with respect to the translational start site of O7 and were named O8pGEX-5' and O8pGEX-3'. This region was chosen as it contained a number of possible of antigenic sites and avoided most of the proline rich area of the protein (aa 509 – 768), as proline rich proteins are often not well tolerated in bacterial expression systems. The area used also included a number of hydrophilic regions as shown by the hydrophobicity plot (Figure 3.3.1.6). Additionally the fact that the region contained a number of putative N and O glycosylation sites (Figure 3.3.1.4 & 5) indicated that this region might be the ectodomain region of O8. O7 was not chosen to raise an antibody, as it was predicted to encode a signal peptide, which would be cleaved away from the rest of the protein.

The region of interest was amplified by PCR using OvHV-2 genomic DNA as a template. The amplified region was ligated into pGEX-3X in frame with the GST sequence using the *Bam*HI and *Eco*RI sites to make the construct O8pGEX (Figure 3.3.4.1). The construct was transformed into *E. coli* bacteria and transformants checked for presence of the inserted O8 DNA by restriction analysis of small-scale preparations of plasmid DNA with *Eco*RI and *Bam*HI. Transformants containing the inserted O8 DNA were then tested for expression of the O8-GST fusion protein.

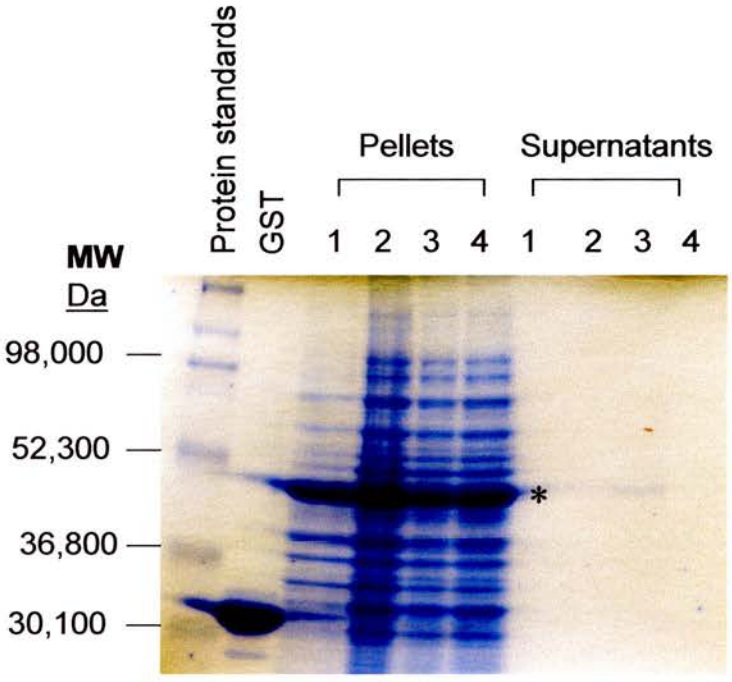
Four samples positive for the O8 insert were analysed for expression of the GST fusion protein. Expression of the GST fusion protein was induced by addition of IPTG to 0.1mM during the exponential growth phase of the culture. The bacteria were harvested 3 hours after induction and lysed by sonication. Both the supernatant and the cell pellet were then analysed by electrophoresis on a 12% SDS-PAGE gel.

Figure 3.3.4.1 O8pGEX Construct Used for Expression of an O8-GST Fusion Protein



A 600bp section of O8 was amplified by PCR using the primers O8pGEX-5' and O8pGEX-3'. The PCR product was digested using *Bam*HI and *Eco*RI and ligated to the vector pGEX-3X. The vector encodes the *Schistosoma japonicum* Glutathione S-Transferase (GST) protein sj26. The vector is designed so that the sequence of interest (O8) is expressed as a GST fusion protein under the control of the *tac* promoter (p*tac*). The Cloning site is followed by translational STOP signals in all three open reading frames. Under normal conditions, expression is repressed by the product of the $LacI^q$ gene, however repression is removed by the addition of the inducer isopropyl β-D thiogalactosidase (IPTG), a lactose analogue. The plasmid contains the ampicillin resistance gene (AmpR) for selection of plasmid containing colonies and a pBR322 origin of replication (Ori).

Figure 3.3.4.2 Analysis of Expression of the O8-GST Fusion Protein



A 621bp sequence of O8 was cloned into the vector pGEX-3X and transfected into the XL-1 blue strain of *E.coli*. Transfected bacteria were grown overnight and induced with 0.1mM IPTG for 3 hours. The bacteria were lysed by sonication on ice and pellet and supernatant fractions were analysed on a 12% SDS-PAGE gel. Proteins were visualised by Coomassie blue staining. The molecular weights of protein standards are indicated, and the position of the O8-GST fusion protein is marked with a star.

The electrophoresed proteins were identified by Coomassie staining of the gel (Section 2.8.2). All samples contained a major product of approximate size 52 KDa predominantly in the pellet fraction (Figure 3.3.4.2). The expected size of the O8 protein was approximately 23 KDa and the GST protein was 30Kda, therefore the 52kDa band was the right size to be the O8-GST fusion protein. The fact that the GST fusion protein was found in the bacterial pellet meant that this protein was insoluble. It was thought that this effect could have possibly been observed due to insufficient sonication of the bacteria, however on repeating this, the GST fusion protein still appeared insoluble (Figure 3.3.4.3A).

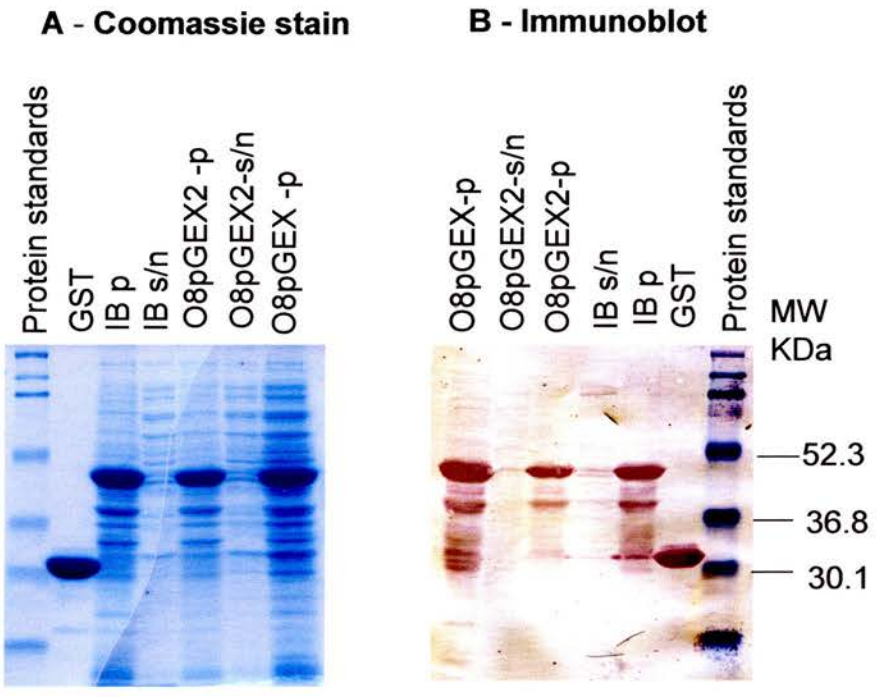
3.3.4.2 Purification of The GST Fusion Protein By Inclusion Body Preparation

Foreign proteins that are overexpressed in *E. coli* and found to be insoluble often form insoluble aggregates called inclusion bodies (Marston, 1986). These are readily isolated by low speed centrifugation and often consist of almost pure aggregations of the denatured form of the foreign protein. The GST fusion protein was therefore purified by means of an inclusion body preparation (Section 2.9.2).

Clone 3 containing the O8pGEX construct was induced with 0.1mM IPTG and incubated for 3 hours at 37°C before harvesting. The cells were then resuspended in inclusion body prep solution and subjected to gentle lysis with lysozyme. Contaminating nucleic acids were removed from the lysate by incubation with DNaseI and the inclusion bodies were removed from suspension by centrifugation at 10,000 x *g*. The inclusion bodies were then washed in inclusion body prep solution containing NP40, to remove contaminating bacterial proteins, before resuspension into a 50% (v/v) solution in PBS. Samples of the supernatant and pellet fractions from the inclusion body preparation were then analysed on a 12% SDS-PAGE gel to determine presence of the O8-GST fusion protein. A protein sample from an induced bacterial strain containing the vector pGEX-3X without an insert was also electrophoresed to show the size of the GST protein.

The O8-GST fusion protein was present in the insoluble inclusion body preparation and not in the supernatant fraction (Figure 3.3.4.3A). A sample of the

Figure 3.3.4.3 Analysis of O8-GST Fusion Proteins Prepared Using Different Methods



A - O8-GST Fusion proteins were analysed on a 12% SDS-PAGE gel and visualised by Coomassie blue Staining

B - The same samples were analysed by western blotting using a goat anti-GST antibody, followed by a secondary biotinylated anti-sheep Ig, and a tertiary streptavidin-AP. Antibody binding was detected by incubation with Sigma FAST BCIP/NBT solution. The molecular weights of protein standards are indicated.

GST : Glutathione S-transferase P: Pellet s/n: Supernatant
O8pGEX : Products from lysis of bacteria transfected with O8pGEX.
O8pGEX2: Products of lysis of O8pGEX transfected bacteria, prepared with extra sonication. IB : Inclusion body preparation from O8pGEX transfected bacteria.

inclusion body preparation was transferred to a PVDF membrane by western blotting (Section 2.8.3) and the membrane incubated with a goat anti-GST antibody at a concentration of 1/1,000, followed by incubation with an anti-sheep Ig conjugated to biotin. The tertiary detection reagent used was a streptavidin conjugated to alkaline phosphatase (streptavidin-AP). Antibody binding was then visualised by use of Sigma FAST BCIP/NBT solution. The positive band was seen in at approximately 52 KDa and smaller bands were also seen at approximately 40KDa and at 30KDa, the same size as the GST band, possibly indicating some breakdown of the protein (Figure 3.3.4.3B). Since particulate antigens are suitable for injection, the inclusion body preparation was used to immunise two New Zealand White rabbits as described (Section 2.9.3).

3.3.4.3 Analysis of Immune Rabbit Sera For The Presence Of Antibodies To The O8-GST Fusion Protein

In order to check that immunisation of the rabbits had been successful, normal rabbit sera (pre-bleed) and post immunisation rabbit sera were tested at different concentrations for the presence of antibodies to the O8-GST fusion protein. A sample of the inclusion body preparation was resuspended in SDS-PAGE buffer and loaded onto a 12% SDS-PAGE gel with only two lanes. The lane of standard width was loaded with the protein molecular weight standard, whilst the wide lane was loaded with the O8-GST inclusion body preparation. The O8-GST fusion protein was therefore electrophoresed in one wide band down the gel.

After electrophoresis the proteins were transferred to a PVDF membrane by western blotting and the membrane was cut into narrow strips. This was to enable incubation of the proteins with different concentrations of primary and secondary antibodies to determine an optimal combination, which showed specific antibody binding, but with minimum non-specific antibody binding. Antibody binding observed using both the normal rabbit serum and in the immune serum was interpreted as non-specific binding. The rabbit serum was used at concentrations ranging from 1/1,000 to 1/10,000. The secondary antibody was a porcine anti-rabbit Ig conjugated to alkaline phosphatase. This was used at concentrations from 1/1,000

to 1/10,000. In each membrane one strip was probed with a polyclonal rabbit anti-GST antibody used at a concentration of 1/500. The secondary antibody used was the porcine anti-rabbit Ig at 1/1,000 concentration. Alkaline phosphatase activity on the membrane was detected by incubation of the strips in Sigma FAST BCIP/NBT solution. All immune serum concentrations demonstrated a specific antibody response to the 52KDa band, (Figure 3.3.4.4). The rabbits had also developed a strong antibody response to a protein of size approximately 24KDa, which the anti-GST antibody did not specifically recognise. It is possible that this protein was a breakdown product of the GST fusion protein. The optimum concentration of primary and secondary antibody for minimising non-specific antibody binding was a serum concentration of 1/2,000 and a secondary antibody concentration of 1/2,000. On the basis of the antibody response shown by western blotting, both rabbits were euthanased and the serum separated from the blood. The sera were aliquoted and frozen at –20°C.

3.3.4.4 Immunofluorescence Analysis of O8 Expression in OvHV-2 Infected Cell Lines Using The Immune Rabbit Serum

In order to attempt to detect expression of the O8 protein in BJ/880 OvHV-2 infected rabbit cells, serum from rabbit B was used in immunofluorescence on acetone fixed cytocentrifuge preparations of the OvHV-2 infected rabbit cell line BJ/880 (obtained from the Moredun Research Institute). The immune rabbit serum was used as the primary antibody. The secondary antibody was a porcine anti-rabbit Ig conjugated to FITC and was used at a concentration of 1/50. Pre-immune rabbit serum was used as a control at the same concentrations as the immune serum using the same secondary antibody. As a control to check for binding of the secondary antibody to the cell, some samples were incubated with PBS as the primary reagent followed by the anti-rabbit FITC as the secondary reagent.

Immunofluorescence analysis using the immune rabbit serum at a concentration of 1/100 showed positive fluorescence in a large proportion of cells (Figure 3.3.4.5). The localisation of the staining appeared to be around the cell surface and in the cytoplasm. Some fluorescence was observed on the cells incubated with normal rabbit serum, however this was not of the same intensity as the

fluorescence seen following incubation with the immune serum. The cells incubated with PBS followed by FITC exhibited only low levels of fluorescence, this suggested that the secondary anti-rabbit FITC was not binding to the BJ/880 OvHV-2 infected rabbit cells. Immunofluorescence staining was performed on paraformaldehyde fixed cytocentrifuged cells, obtained from the Moredun Research Institute, as paraformaldehyde fixing of cells generally preserves the cellular architecture better than acetone fixation. No positive fluorescence was seen in these samples. This might have been because paraformadehyde treatment can destroy some protein epitopes, therefore the optimum method of fixing has to be determined for the antigen to be detected. The positive fluorescence observed in the OvHV-2 cells incubated with immune serum as compared to samples incubated with pre-immune serum suggested that a high proportion of cells were expressing the O8 protein. The use of control non-infected cells was considered to be necessary to reinforce the validity of this result.

3.3.4.5 Immunofluorescence Analysis of PHA Treated Control Rabbit Lymphocytes

The OvHV-2 infected rabbit lymphocytes are a lymphoblastoid phenotype (Reid *et al.*, 1983). It was considered important to try to make a non OvHV-2 infected negative control for immunofluorescence analysis using the immune rabbit serum. The most appropriate control for use was considered to be activated normal rabbit lymphocytes. Lymphocytes were separated from rabbit blood by Ficoll density centrifugation using Lymphoprep (Section 2.10.4). The lymphocytes were then incubated for 48 hours with 7.5μg/ml PHA. This resulted in the cells displaying an activated phenotype, as shown by the cells adhering to each other and forming irregular shapes (Section 2.10.5). The cells were harvested and washed in PBS, after which they were cytospun onto slides as described (Section 2.10.1). The cytospun activated lymphoblasts were then fixed in 100% (v/v) acetone and were incubated with the immune rabbit serum followed by the anti-rabbit FITC in the same way as was carried out in the BJ/880 OvHV-2 infected lymphocytes.

The PHA treated control rabbit lymphocytes showed generally low levels of fluorescence in both the immune serum incubated and pre-immune serum incubated

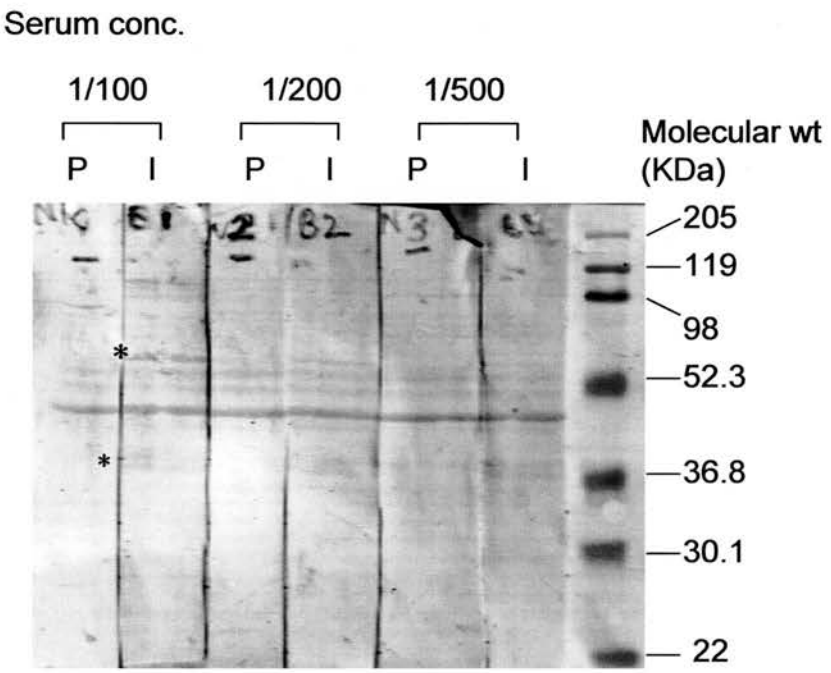
samples (Figure 3.3.4.5). A small number of cells in the immune serum treated samples did, however exhibit fluorescence of a similar staining pattern and intensity to the OvHV-2 infected rabbit cells. The samples incubated with PBS followed by anti-rabbit FITC exhibited cell surface and intracellular fluorescence in a small number of cells. This staining was of higher intensity than that seen on PBS and anti-rabbit FITC staining of OvHV-2 infected lymphoblastoid cell lines. The fluorescence seen on PBS/anti-rabbit FITC incubated control rabbit lymphocytes suggests that some of the fluorescence seen after incubation of these fixed cells using immune and pre-immune serum may have been caused by binding of the secondary anti-rabbit FITC antibody to the cells. It was also observed that whilst the OvHV-2 infected LCLs were known to be only T lymphocytes, PHA treated rabbit lymphocytes would also contain B lymphocytes, and binding of the primary antibody to Fc receptors on these cells may cause problems with background fluorescence.

3.3.4.6 Western Blotting Analysis Using Anti-O8 Immune Rabbit Serum

OvHV-2 infected BJ/880 rabbit cells were analysed by western blotting to determine the size of the O7/O8 protein. BJ/880 cells were lysed in sample buffer and electrophoresed on a 12% SDS-PAGE gel. The electrophoresed proteins were transferred to a PVDF membrane and the membrane was cut into strips. The strips were incubated with varying concentrations of the primary antibody, which was immune rabbit serum, or pre-immune rabbit serum. Concentrations varying from 1/100 to 1/5,000 were used. The strips were then incubated with the secondary anti-rabbit-AP conjugate at a concentration of 1/5,000 and antibody binding was detected by incubation with Sigma FAST BCIP/NBT solution. After incubation with the detection reagent a number of bands were detectable, a 53KDa band and two smaller bands of size approximately 37KDa and 38KDa seemed to be recognised by the immune serum but not the pre-immune serum at concentrations of 1/100 down to 1/1,000 (Figure 3.3.4.6).

Western blotting was repeated using PHA treated rabbit lymphocytes as negative control cells (Section 2.10.5). A number of bands were observed from

Figure 3.3.4.6 Western Blotting Analysis of OvHV-2 Infected Rabbit Cells Using the Anti-O8 Immune Serum



Cell lysate of the OvHV-2 infected rabbit cell line BJ/880 was electrophoresed on a 12% SDS-PAGE gel and the proteins transferred to a PVDF membrane. The membrane was cut into strips and the strips incubated with different concentrations of immune (I) or pre-immune (P) rabbit serum. The strips were then incubated with a secondary anti-rabbit alkaline phosphatase conjugate and antibody binding was visualised using Sigma FAST BCIP/NBT solution. The concentrations of sera used are shown above the strips and the molecular weight of protein standards indicated. The position of bands of interest described in the text are marked with stars.

analysis of lysates from infected cells but not in the control rabbit lymphocyte cell lysates. Electrophoresed cell lysate from equal numbers of the control lymphocytes and BJ/880 was analysed by Coomassie staining. This showed that the amount of protein in the BJ/880 cells was far greater than the amount in the same number of control lymphocytes, therefore it was concluded that the differences on western blotting analysis were be due to different amounts of protein on the SDS-PAGE gel. This experiment was not repeated due to time constraints and the lack of rabbit blood for isolation of lymphocytes. The initial western blotting analysis of cell lysates from OvHV-2 infected rabbit cells showed that the anti-O8 antiserum was recognising three proteins of 53KDa, 37KDa and 38KDa that were not recognised by the pre-immune rabbit serum. This suggested that the binding of the serum to these protein species was specific and that these proteins might represent different forms of the O8 protein.

3.3.4.7 Analysis of Ovine And Bovine Sera For The Presence of Anti-O8 Antibodies

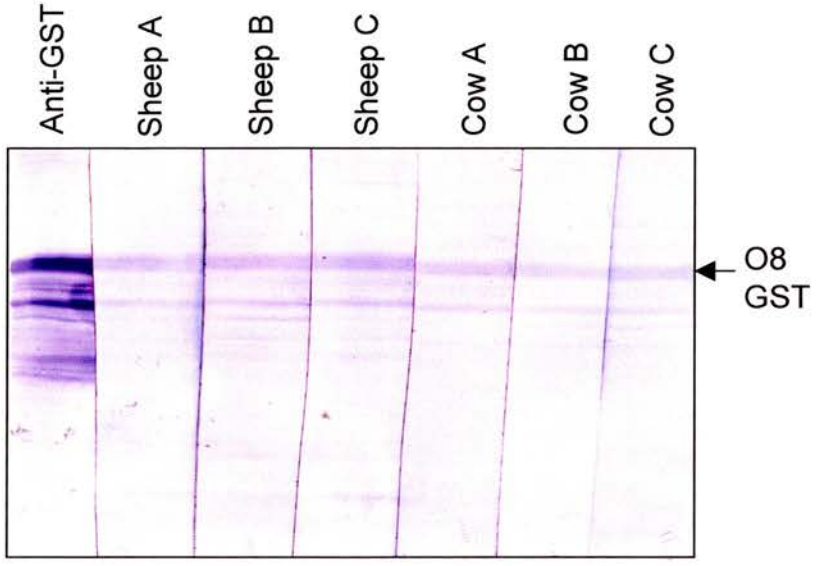
Sheep are considered to be the natural host of OvHV-2 and since sheep have been shown to transmit disease to other susceptible animals, it is thought that the full cycle of viral replication with production of viral particles must occur in sheep. It has been shown that adult sheep have antibodies that cross-react with AlHV-1 proteins (Herring *et al.*, 1989), therefore these antibodies are expected to react with OvHV-2 proteins. Since a major part of the antibody response to EBV is generated against the envelope glycoprotein gp350 (Thorley-Lawson & Poodry, 1982) and O8 is a predicted to encode a similar envelope glycoprotein, it was hypothesised that sheep might develop an antibody response to O8.

In order to test sheep sera for antibodies to O8, the O8 inclusion body prep was electrophoresed on a 12% PAGE gel with a wide lane in the same way as was carried out to test rabbit sera for the presence of antibodies (Section 3.3.4.3). The proteins were then transferred to a PVDF membrane by western blotting. Since sheep antibodies often display a high degree of non-specific binding, the membrane was incubated in a blocking solution containing 5% (w/v) non-fat dried milk and 5%

(v/v) equine serum. This solution was also used for dilution of antibodies. The membrane was cut into strips and the strips incubated with sera at a concentration of 1/50. The serum samples were taken from three adult sheep (A, B and C), two cattle that had recovered from malignant catarrhal fever and one normal cow (presumed not to have antibodies to OvHV-2). As a positive control, one strip was incubated with a polyclonal rabbit anti-GST antibody.

The strips were incubated with the sera for two hours and with the secondary and tertiary reagents as described (Section 2.8.4). A band was detected in all samples at the position of the GST fusion protein, as shown by binding of the anti-GST antibody (Figure 3.3.4.7), however, since binding was also seen on the sample from the non-infected cow, this binding was considered to be non-specific. In order to decrease non-specific antibody binding, a protocol involving the use of Tween 80 to a concentration of 0.5% and a "high salt" hybridisation and blocking solution as described in (Section 2.8.4), however this had no effect on the amount of non-specific binding. It was hypothesised that sheep may be exposed to only small amounts of the O8 protein during infection, therefore the antibodies may be of low titre. Increasing the serum concentration to 1/20 and 1/10 did not however, result in positive bands on western analysis. It was concluded, therefore that there was no evidence that sheep or infected cattle develop specific antibodies to the section of O8 protein tested.

Figure 3.3.4.7 Western Blotting Analysis of the O8-GST Fusion Protein Using Bovine and Ovine Serum



A sample of O8-GST inclusion body preparation electrophoresed on a 12% SDS-PAGE gel. The proteins were then transferred to a PVDF membrane by western blotting and the blot cut into strips. The strips were incubated with primary antibodies, which were rabbit anti-GST antibody (Anti-GST), serum from three normal sheep (A, B and C), serum from cow A (recovered MCF case), cow B (recovered MCF case) and cow C (OvHV-2 negative cow). The secondary antibody to the bovine or ovine serum was a biotinylated anti-sheep antibody, whilst the secondary for the anti-GST was an anti-rabbit-AP antibody. Strips incubated with bovine or ovine sera were then incubated with a tertiary streptavidin-AP and antibody binding in all strips was detected using BCIP/NBT solution. The position of the O8-GST fusion protein is marked on the right side of the image.

Chapter Four: Discussion

4.1 OvHV-2 Replication

4.2 Generation of Sequence of the OvHV-2 Genome

4.3 Sequence Analysis of the OvHV-2 Genome

4.4 Characterisation of the O7 and O8 ORFs

4.5 Conclusions

4.0 Discussion

Research into the pathogenesis of OvHV-2 has been hampered by the lack of a tissue culture system supporting productive replication of the virus. Compared to AlHV-1, relatively little is known about OvHV-2. At the start of the work the only available virus sequence was the OvHV-2 ORF49 sequence, derived from screening of an OvHV-2 expression library (Coulter & Reid, 2002) and the OvHV-2 ORF 75 sequence, which was the first OvHV-2 sequence identified (Bridgen & Reid, 1991). OvHV-2 infected rabbit and cattle cell lines were available and these had been the source of all the virus sequence to date. Some OvHV-2 infected cell lines transmitted disease, however the mechanism of viral persistence in these cell lines was unknown. The aims of the project were initially to further characterise the OvHV-2 infected cell lines in terms of viral persistence. This was to be performed as a starting point before construction of a cosmid library from OvHV-2 infected cell lines, in order to derive more viral sequence. It was then envisaged that having derived more OvHV-2 viral sequence, a selected area of the genome of interest would be studied in greater detail.

4.1 OvHV-2 Replication

Cell lines propagated from the tissues of cases of SA-MCF have been shown to transmit disease and viral DNA with homology to that of AlHV-1 has been identified in the cell lines. This led to the hypothesis that the cell lines carry the virus OvHV-2. This work was performed in order to further characterise OvHV-2 infected cell lines in terms of the amount of virus in the cell lines and the mode of viral persistence.

The copy number of viral genomes in the OvHV-2 infected cells was determined by Southern blotting and the results show that the cells contain on average 50 copies of viral genome per cell. This finding was similar to the amount of virus found in other gammaherpesvirus infected cell lines, as the EBV infected Burkitt's Lymphoma cell line Raji contains 50 copies of latent viral genome per cell (Adams & Lindahl, 1975) and the MHV-68 infected S11 cell lines contain on average 40 copies of virus per cell (Usherwood *et al.*, 1996). This work was carried out in preparation for the construction of a cosmid library from DNA extracted from OvHV-2 infected cell lines. The finding of an average of 50 copies of viral genomes per cell meant that the cell lines contained sufficient virus DNA to enable

construction of a cosmid library, as this had been performed using the DNA of Turkey Herpesvirus cell lines, which contain similar numbers of viral genomes (Reilly & Silva, 1993a).

Until this point, little was known about the state of the OvHV-2 viral genome in OvHV-2 infected cell lines. Although some OvHV-2 infected cell lines transmit disease (Reid *et al.*, 1983), infectious virus had not been identified in the cell lines, and the mechanism of viral persistence in the cell lines was not known. It was of interest to determine if there was evidence of viral replication in the OvHV-2 infected cell lines, as this would suggest that viral particles might be produced. Gardella gel analysis was used to determine the state of the viral genome in OvHV-2 infected bovine and rabbit cell lines. Gardella gel analysis is an established technique for the analysis of herpesviral DNA. It has been used for detection of the latent form of EBV DNA in the peripheral blood cells of healthy individuals (Decker *et al.*, 1996a) and for detection of KSHV DNA in infected tissues (Decker *et al.*, 1996b).

On analysis of the OvHV-2 infected rabbit cell lines, an obvious smear of linear DNA was detected, whilst the upper band indicating circular viral DNA was of lower intensity. On analysis of the OvHV-2 infected bovine cell line BJ/1035, the upper band of circular DNA was of higher intensity than that seen on analysis of rabbit cell lines. A lower smear of linear DNA was present but was of very low intensity. The presence of circular DNA is evidence of episomal viral DNA as is found in latently infected cells, whereas the presence of linear viral DNA is evidence of viral DNA replication. The results suggested that a substantial amount of linear DNA was present in OvHV-2 infected rabbit cells as compared to bovine cells. This suggested that a sizeable proportion of the OvHV-2 infected rabbit cells analysed contain replicating viral DNA, although some cells are latently infected. In the bovine cell line, evidence suggests that cells are predominantly latently infected with low levels of viral DNA replication.

Once optimised Gardella gel analysis of OvHV-2 infected cell lines was simple and the results were consistent and reproducible. Use of the control rhadinovirus infected cell lines S11 and BCP-1 reinforced the relevance of the results from analysis of OvHV-2 infected cell lines. Episomal DNA identified on analysis of the rabbit and cattle cell lines migrated in a similar manner to episomal DNA from

the S11 and BCP-1 cell lines. The smear of linear viral DNA found on analysis of the OvHV-2 rabbit cell lines was similar to that seen on analysis of the MHV-68 infected cell line S11, therefore it was concluded that the lower and upper bands seen on analysis of OvHV-2 infected rabbit cells did represent the linear and circular forms of the viral genome.

The easily visible smear of linear DNA identified on analysis of OvHV-2 infected rabbit cells is suggestive of a substantial amount of viral replication, however it gives no indication of the number of cells in which DNA replication is taking place. The S11 cell line contains up to 20-30% of cells productively infected, whilst the remainder are latently infected (Usherwood *et al.*, 1996). It is tempting to suggest that a similar proportion of cells in the BJ/2222 cell line might be productively infected, however this could only be determined by *in situ* hybridisation of the OvHV-2 infected cell lines using probes for genes known to be expressed in lytic or latent infection. The BCP-1 cell line contains approximately 1% of cells lytically infected with KSHV. The intensity of the lower band of linear DNA seen on analysis of OvHV-2 infected bovine cells is similar to that seen on analysis of the KSHV infected BCP-1 cell line, it may be that a similar proportion of cells in the OvHV-2 infected bovine cell line contain replicating viral DNA, however further analysis would be required to determine this.

There is a small possibility that some of the linear viral DNA was produced by nicking of episomal DNA. Comparison of the OvHV-2 infected cells with the same cell line treated with antiviral agents such as acyclovir would be required to prove that this was not the case, however the fact that linear DNA was not detectable in the same amounts in either the KSHV infected cell lines or in the cattle cell lines, which were processed in exactly the same way as the rabbit cell lines, suggests that the linear DNA in the cell lines is not an artefact. The strong evidence of substantial amounts of linear viral DNA in rabbit cells lines prompted the analysis of these cell lines for evidence of the expression of lytic cycle genes.

OvHV-2 infected cells were analysed for the presence of lytic viral transcripts by northern blotting and RT-PCR. The expression of both immediate early viral genes (ORF 57) and late viral genes (ORF 75) was shown in the OvHV-2 infected rabbit cell line BJ/2222 by RT-PCR. It was not possible to detect the expression of

OvHV-2 transcripts by northern analysis and treatment of the rabbit cell line BJ/2222 with TPA did not increase viral gene expression to the level of detection. It was not possible to perform further analysis on the bovine cell line BJ/1035 as the cell line died soon after completion of the Gardella gel analysis. Generally the OvHV-2 infected cell lines were short lived and the availability of OvHV-2 infected cell lines for analysis was a constant constraint.

The expression of both the early gene ORF 57 and the late gene ORF 75 was evidence that both early and late viral gene expression was taking place in some of the OvHV-2 infected rabbit cells. Since the PCR primers did not span splice sites, care was taken to ensure that the products were as a result of PCR amplification of cDNA and not of contaminating genomic DNA. The samples were incubated with DNaseI before the reverse transcription reactions. A sample of each RNA was used to set up an RT reaction without enzyme, so that any amplification products from this reaction would show that the products were as a result of genomic DNA contamination. Reverse transcription reactions performed using cytoplasmic RNA were not successful. This was possibly due to the fact that this method of isolation required the use of phenol : chloroform and contamination of RNA samples with phenol can affect the reverse transcriptase enzyme, therefore subsequent RT reactions were performed on RNA extracted by a different method.

The failure to detect expression of lytic cycle genes in OvHV-2 infected cell by northern analysis suggests that levels of the lytic gene transcripts in the cell lines were low. This could be because expression of ORF 57 and ORF 75 was low, or that the transcripts had a very short half life and were not allowed to accumulate in large amounts. The failure to increase expression of lytic cycle genes in OvHV-2 infected cell lines to detectable levels is in contrast to KSHV infected cell lines and EBV infected cell lines, which both show an increase in the proportion of cells undergoing lytic gene expression by treatment with TPA (Renne *et al.*, 1996b) (Lin *et al.*, 1983). Some HVS infected rabbit cell lines do not produce infectious virus, however the expression of lytic transcripts can be increased by treament with TPA (Ablashi *et al.*, 1985). A HVS infected human T cell line which did not produce infectious virus was found to increase the expression of transcripts of the transforming protein STP-C on treatment with TPA, however the expression of lytic transcripts was not increased to

detectable levels (Fickenscher *et al.*, 1996). It is possible therefore that treatment of OvHV-2 infected cell lines with TPA had effects on genes other than the lytic transcripts examined. The probability of detecting viral transcripts by northern analysis could have been increased by the use of poly A enriched RNA, however this was not possible at the time because more OvHV-2 infected cells were not available.

Evidence of the expression of both early and late lytic viral genes in OvHV-2 infected rabbit cells suggested that the full lytic cycle might be taking place in some cells, therefore the cells were examined for the presence of viral particles. It was not possible to detect viral particles by transmission electron microscopy of fixed OvHV-2 infected rabbit cells. Cell lysates from the OvHV-2 infected rabbit cell line BJ/2222 were examined for the presence of viral particles. From these cell lines, a herpesvirus capsid was visualised. This finding is significant as up until now it has not been possible to detect the presence of any components of viral particles in OvHV-2 infected cell lines. EM analysis of cell lysates was carried out as an alternative method to direct EM of fixed cells, as this method is a way of concentrating the cell contents. This makes detection of any virus particles more feasible; therefore the method is particularly suited to cell lines in which only small numbers of particles were present. The method was the same as that used to visualise KSHV viral particles in the KSHV infected cell line BC-3 (Arvanitakis *et al.*, 1996).

Although the electron micrograph clearly shows an icosahedral capsid typical of a herpesvirus, the viral envelope is not obvious and it is possible that the image is that of a non-enveloped particle. It is not known if this is an artefact of processing, or if production of fully enveloped viral particles does not occur in OvHV-2 infected rabbit cells. Although Gardella gel analysis seemed to suggest that substantial amounts of linear DNA was present in OvHV-2 infected rabbit cell lines, the fact that it was not possible to detect expression of either ORF 57 or ORF 75 by northern analysis suggests that expression of lytic cycle genes is low. This might mean that only a small proportion of cells are undergoing lytic cycle replication and production of viral particles is low. Since a virus capsid has been visualised but no envelope, it is also possible that DNA replication and expression of early and late viral genes occurs, but the viral lytic cycle is not complete and production of mature enveloped capsids does not occur. The fact that the OvHV-2 infected cells transmit disease,

however suggests that the virus in the cells does retain the ability to undergo lytic replication given the right conditions.

4.2 Generation of Sequence of the OvHV-2 Genome

Research into the pathogenesis of OvHV-2 has long been hampered by the lack of a tissue culture system for growing the virus. This is in contrast to AlHV-1, which can replicate in tissue culture cells and whose genome has been completely sequenced. The fact that some OvHV-2 infected cell lines transmit disease suggested that the complete viral genome was present in these cell lines and that these cell lines might be a source from which the viral genome could be cloned. The OvHV-2 genome was predicted to be similar in size to that of AlHV-1, therefore was expected to be approximately 150kb in size. In order to sequence the OvHV-2 genome, a cosmid library was constructed using genomic DNA extracted from the OvHV-2 infected bovine cell line BJ/1035. The library was screened using an OvHV-2 specific probe, which resulted in the identification of the first virus containing cosmid, named C75. Walking probes were made based on the sequence at the ends of the virus containing cosmids and a series of 5 cosmids was identified which spanned most of the viral genome (Figure 3.2.3.1). At the left hand end of the genome the cosmid C8 extended into O2. At the right hand end of the genome the cosmid C75 extended to the right of ORF O9. It was not possible to obtain cosmid clones containing virus sequence to the right of cosmid C75 or to the left of cosmid C8.

Cosmids were used as cloning vectors due to their large cloning capacity. Vectors such as bacteriophage vectors are often used for construction of genomic libraries, as they allow DNA fragments as large as 20kb to be cloned. Cosmid vectors, however, are an improvement on bacteriophage vectors, in that they can package inserted DNA of up to 40kb in size and therefore require the screening of a smaller number of clones to identify the sequence of interest. Cosmid cloning has been used in the past for genomic walking of eukaryotic DNA (Wahl *et al.*, 1987) and also in the sequencing of virus genomes. Cosmid cloning was used in the sequencing of the turkey herpesvirus genome (Reilly & Silva, 1993a). The genome of KSHV was identified by screening cosmid and phage libraries constructed using DNA extracted from the KSHV infected cell line BC-1 (Russo *et al.*, 1996). In both

these examples, the viral DNA genome was cloned along with an excess of cellular DNA. This illustrates that this approach is a valid way of sequencing viruses that do not grow well in tissue culture.

The vector SuperCos 1MW had been modified for cloning in HSV. The cloning site had been replaced by a synthetic oligonucleotide sequence and the T7 and T3 bacteriophage promoter sequences were no longer present. These sequences were present so that RNA walking probes could be synthesised against the sequence of each end of the vector using bacteriophage DNA dependant RNA polymerases. The lack of these sequences meant that walking probes were instead made by PCR amplification of known sequence at each end of the cosmid insert. Sequencing of the ends of the inserted DNA was therefore necessary. This however gave the advantage that in most cases it confirmed that the new sequence was viral, before using this to screen the library. False positive clones did prove a problem and probing the library with the probe C75R amplified from a cosmid DNA template resulted in a false positive clone which was later shown to be non viral. The problem was thought to have been caused by the presence of the template cosmid in the probe. Ensuring that template was removed from the PCR product to be used as a probe solved this problem. This shows that the presence of sequence such as cosmid in the probe, which would have homology to all cosmid sequences, can result in the generation of false positives. The use of RNA probes synthesised by *in vitro* transcription can also lead to problems with false positive clones, as all probes have sequence complementary to cosmid at the 5' end.

This cosmid library generated contained 16,000 transformants. The estimation of the average copy number of the viral genomes in OvHV-2 infected cattle cell lines was 50 copies per cell. Assuming two copies of every cellular gene, this meant that there was on average a 25 fold excess of unique viral sequences over unique cellular sequences. It was calculated that 13,818 transformants would be required to have a 99% chance of finding a viral sequence in the library. This meant that the library should have been representative of each sequence. The fact that screening the cosmid library resulted in the identification of clones covering a large proportion of the viral genome indicated that the library was quite comprehensive, however it was not possible to obtain clones to the right of the C75 clone, or to the

left of the C8 clone. The BJ/1035 bovine cell DNA was used to construct the cosmid library because this was shown to contain viral DNA predominantly in the circular episomal form. Use of circular herpesviral DNA in cosmid cloning has resulted in the identification of clones spanning the terminal repeats (Andrew Davison, Pesonal Communication), this type of clone has, however, not been identified in this case.

There could be a number of reasons for the failure to extend the viral sequence further towards the viral terminal repeat sequences. Bacterial clones carrying recombinant cosmids grow at different rates and it is possible that a proportion of clones did not grow well during amplification of the library. The library was amplified to increase the number of bacteria carrying each transformant. This is to ensure a stock of cosmid containing bacteria large enough to screen the library several times. Amplification of cosmid libraries can be performed either by replica plating and storage of the library on filters at –70°C (Hanahan & Meselson, 1980), or by transduction of the packaged cosmids into bacteria and growing the bacteria for a few generations in liquid culture. The cosmids were amplified in liquid culture, as this protocol was supplied with the SuperCos 1 vector.

This method is easier and quicker than replicating the bacteria on filters, however it can lead to a change in the composition of the library during replication, as some cosmid containing bacteria grow better in the cultures than others. This is a problem that would not have been encountered if the library was amplified by replica plating. It is also possible that the inserted DNA in some clones was unstable. Recombination between repeated elements as would be found in clones containing terminal repeat sequence can lead to loss or rearrangement of cloned sequence. Evidence that some cosmid containing clones were unstable was found on analysis of C57 cosmid DNA (Section 3.2.1.5) when some cosmid clones seemed to digest to a disproportionally large amount of vector DNA. This suggested that some bacteria had lost their inserts whilst growing in culture. If this occurred during amplification of the cosmid library it would lead to the loss of cosmid clones and the library having a lower titre than the calculated titre.

Since screening the library using the C75R probe and the A2 probe resulted in no new clones, alternative methods of extending the virus sequence were required. It was hypothesised that the virus DNA extracted from the OvHV-2 infected bovine

cell line contained the full virus sequence, therefore this DNA was used as a source to extend the viral sequence beyond that obtained by cosmid cloning. DNA extracted from the OvHV-2 infected bovine cell line BJ/1035 was used as a template for splinkerette PCR. In the first round of splinkerette PCR, OvHV-2 DNA was digested with one of 4 restriction enzymes, *Bam*HI, *Xba*I, *Hin*dIII, or *Eco*RI and ligated to a "splinkerette" linker molecule. Splinkerette PCR amplification of the ligated *Hin*dIII digest using primers designed to extend the sequence from O2 resulted in the generation of clone splink1. This clone was 1.2kb in size and extended to the left of the cosmid clone C8. Sequence analysis of clone splink1 showed that it contained the remainder of the O2 virus sequence continuing from clone C8. PCR amplification of the *Bam*HI digested DNA using a primer designed to extend sequence from the new splink1 sequence resulted in the generation of clone splink2. This clone was 900bp in size. Sequence analysis showed that although this clone did not contain sequence homologous to known viral sequences, the sequence overlapped with splink1.

PCR amplification of the *Bam*HI digested OvHV-2 DNA using a primer designed to amplify sequence from the right hand end of cosmid C75R resulted in the 200bp clone splinkC75R. Sequence analysis of SplinkC75R showed that the sequence overlapped with the end of cosmid C75R, however the sequence did not have any homology with known virus sequences. Attempts to extend sequence beyond the splink2 sequence at the left hand end and the splinkC75R sequence at the right hand end, by splinkerette PCR using the original four OvHV-2 DNA digests was not successful. Splinkerette PCR was therefore repeated using DNA digested with the enzymes *Sal*I, *Bss*HII, *Xma*I, *Bgl*II and *Nhe*I. No new virus clones were obtained using these digests. The technique of splinkerette PCR advanced the known OvHV-2 sequence approximately 2.1kb at the left hand end of the genome, towards the terminal repeats and 200bp at the right hand end of the genome. None of the clones contained terminal repeat sequence, however.

The technique of splinkerette PCR was used because it enabled use of the OvHV-2 genomic DNA to extend the sequence generated by cosmid cloning. The use of "splinkerette" linker molecules enabled PCR amplification of stretches of DNA where sequence information was only available at one end. Splinkerette PCR was originally used for genome walking (Devon *et al.*, 1995). The vectorette and

splinkerette methods have been used for the isolation of end fragments of length up to 3kb from YAC recombinants (Riley *et al.*, 1990) and in PCR walking (Arnold & Hodgson, 1991).

Splinkerette PCR was used rather than vectorette PCR because the technique has been modified to minimise amplification from unligated linker molecules. This was a major cause of non-specific amplification in vectorette PCR. The disadvantage of using splinkerette PCR for genome walking as compared to cosmid cloning is that the walking steps taken are much smaller, however since it was estimated that the remaining sequence to the terminal repeats at each end of the genome may be as little as 1kb this was not a major concern. It was considered that once the sequence of one terminal repeat unit has been obtained, the number of terminal repeats could then be estimated by Southern blotting.

This method of cloning is dependant on the existence of the restriction site of choice within a reasonable distance of the known sequence, such that this sequence can be amplified by PCR. When new PCR products could not be amplified from the original four DNA digests used (*Bam*HI, *Eco*RI, *Hin*dIII, *Xba*I), it was hypothesised that this was because the sequence to be amplified was GC rich terminal repeat sequence and did not contain many restriction sites for the enzymes used to digest the genomic DNA. The five additional enzymes used to digest the OvHV-2 genomic DNA (*Bss*HII, *Nhe*I, *Xma*I, *Sal*I, *Bgl*II) were used because their recognition sites were GC rich and therefore it was expected that they might cut in the terminal repeat sequence. It was considered that the most likely reason that no products were generated by PCR amplification using these enzymes was a lack of restriction sites close enough to the known sequence.

The PCR amplification was carried out using *Taq* DNA polymerase and it is possible that the distance to the restriction sites was too great for amplification using this enzyme, therefore amplification using an enzyme designed for the amplification of long templates, such as the Expand Long Template PCR System (Roche) might have resulted in the generation of new products. It is not possible to use positive controls for this technique of PCR, therefore it was not possible to optimise the conditions for use of the primers. The primers were designed to have similar annealing temperatures and repetitive sequence was avoided when designing the

primers, to avoid the formation of primer dimers. The PCR program used was a combined "hotstart" and "touchdown" program, as used by (Devon *et al.*, 1995). The "hotstart" part of the program was designed to reduce mis-priming at the start of the PCR cycle and has been shown to improve the yield of in amplification of low copy number templates (Chou *et al.*, 1992). The touchdown part of the program was designed to prevent spurious priming during amplification and minimise the need to optimise PCR reactions (Don *et al.*, 1991). This PCR program had been successful in the first two rounds of PCR; therefore the use of a long PCR enzyme and digests with different restriction enzymes may have been a way to obtain further products. The availability of OvHV-2 genomic DNA from the BJ/1035 cattle cell line was limited, however, as the cell line had died, therefore this was a further constraint.

The use of splinkerette PCR advanced the OvHV-2 sequence approximately 2.1kb at the left hand end of the genome and 200bp at the right hand end of the genome. Failure to advance further and the lack of OvHV-2 DNA for further enzyme digestions prompted the use of a different approach to extend the sequence. It has been shown by Gardella gel analysis that the viral DNA in the OvHV-2 infected bovine cell line BJ/1035 was predominantly in the circular episomal configuration (Section 3.1.2), therefore long distance PCR was used to try to extend the sequence over the terminal repeats from the right hand end of the genome to the left. The PCR reactions were performed using the Expand Long Template PCR system. Initial PCR amplification using an annealing temperature of 65°C and an extension time extending from 20 to 25 minutes did not result in the generation of any PCR product. Repeat of this PCR reaction using internal primers, a reduced annealing temperature of 62°C, an increased extension of up to 30 minutes and an increased magnesium concentration, resulted in a high molecular weight smear of PCR product. A repeat of the PCR reaction again using internal primers resulted in a number of bands, the largest of which was approximately 1.3kb in size. This 1.3kb band and a 650bp band were cloned into a TA vector and sequenced, however the sequence did not overlap with existing virus sequence and had homology to cellular DNA sequences. It was concluded that the PCR product had resulted from spurious amplification of bovine cellular DNA.

Gammaherpesvirus genomes contain variable numbers of terminal repeat sequences. HVS contains 30 – 35 copies of a 1.5kb terminal repeat sequence (Albrecht *et al.*, 1992a) and AlHV-1 contains 20 – 25 copies of a repeated sequence of approximately 1.1kb. It was estimated from the layout of the AlHV-1 genome that the distance from the known sequence of OvHV-2 into the terminal repeat sequence may have been less than 1kb at each end of the genome. The total distance over the terminal repeats may therefore have been from 25 – 30kb. It was anticipated that the 1.1kb and 650bp products were too small to contain products of a PCR amplification reaction spanning the terminal repeats, however it was hypothesised that it may have been possible to obtain short products if a viral genome containing only one terminal repeat sequence was amplified.

It was known that PCR amplification of genomic DNA over 12kb and especially over 20kb requires extensive optimisation in terms of template quality, magnesium concentration, denaturation conditions and nucleotide concentration. The elongation times used should have been adequate, as it was recommended by the manufacturer of the Expand Long Template PCR System that an elongation time of 20 minutes should have been adequate to amplify up to 30kb, whilst an extension time of 30 minutes was recommended to be adequate to amplify up to 40kb. Template quality is very important in long distance PCR. The high molecular weight DNA used as a template for PCR amplification was analysed by gel electrophoresis and was of high quality, however high molecular weight DNA does shear easily and sheared template may have been a reason why it was not possible to amplify over the terminal repeats.

Increasing the magnesium concentration in repeats of first round PCR did not result in a visible PCR product and these PCR reactions were not repeated using internal primers due to time constraints. The first round of PCR amplification must have resulted in the generation of a small amount of non-specific PCR amplification products. The reduction in annealing temperature may have improved the conditions for their amplification in the second round of PCR using internal primers. If time had allowed, further attempts at long distance PCR would have been carried out using a more logical optimisation protocol, since using a different PCR protocol for PCR amplification of the original reaction meant that it was unclear if repetition using the

same conditions as in the first round would have resulted in the production of a specific amplification product.

A number of approaches have been used for generation of new OvHV-2 virus sequence, however it has not been possible to complete the virus sequence up to the viral terminal repeats. Further attempts at completing the virus sequence might involve re-optimisation of PCR across the viral terminal repeats, or repeat of splinkerette PCR using different enzymes for digestion of the OvHV-2 genomic DNA. Another approach would be to use a method that does not rely on the existence of the correct restriction site. This could be possible if PCR amplification of linear viral DNA was performed. This would require the use of blunt ended ligation of splinkerette linker molecules to linear OvHV-2 genomic DNA which might be expected to be found in OvHV-2 infected rabbit cell lines.

4.3 Sequence Analysis of The OvHV-2 Genome

It was shown by cross hybridisation with AlHV-1 DNA that the agent causing SA-MCF is highly related to AlHV-1 (Bridgen & Reid, 1991). Comparison of the recently derived sequence of OvHV-2 glycoprotein B (gB) with the gB sequence of other herpesviruses also suggested that OvHV-2 and AlHV-1 were related, but distinct herpesviruses (Dunowska *et al.*, 2001). Analysis of the OvHV-2 sequence cloned from cosmids has confirmed these assumptions. The total OvHV-2 sequence cloned was 127,800bp. The conserved open reading frames are arranged collinear to HVS, in a similar manner to AlHV-1. The fact that many of the AlHV-1 "A" genes, which were considered to be unique to AlHV-1 are also conserved in OvHV-2 confirms the relatedness of the two viruses. Since the full sequence of AlHV-1 was published, sequence from the porcine herpesvirus PLHV-1 has also been published (Goltz *et al.*, 2002). This virus also contains homologues of the AlHV-1 "A" genes, indicating that these three viruses are highly related. All the OvHV-2 ORFs with a homologue in AlHV-1 show the greatest similarity to AlHV-1, however ORF9 (DNA polymerase) shows highest homology with ORF9 of Caprine herpesvirus-2 (CprHV-2) (Li *et al.*, 2001b). This is the only open reading frame of CprHV-2 that has been sequenced, so it is possible that the rest of this virus could be as similar to OvHV-2.

The open reading frame O2 is similar to A2 of AlHV-1 and both ORFs are predicted to be spliced. The O2 gene contains a leucine zipper motif, therefore may function as a transcriptional regulator. The O3.5 gene is located in the same position as the AlHV-1 gene A4. Both ORFs are predicted to contain signal sequences, however the ORFs are not homologous and the possible function is unknown. The block of ORFs from ORF6 to 9 are all highly conserved and encode genes involved in viral DNA replication. All the ORFs involved in viral DNA replication are generally highly conserved, especially among rhadinoviruses. Many of the capsid and tegument proteins are also highly conserved, for example the capsid protein vp23 encoded by ORF 26 shares 83% amino acid identity with AlHV-1 and 48% with HVS. The gB and terminase genes are highly conserved and are used for phylogenetic analysis of herpesviruses.

In common with AlHV-1 and PLHV-1 the genome of OvHV-2 contains a gap between ORF 11 and 17, which does not contain any predicted open reading frames. This region in KSHV and HVS contains homologues of cellular genes. In KSHV it contains cellular homologues such as an IL-6 homologue and homologues of the macrophage inhibitory proteins MIPI and II. In HVS ORF14 encodes a putative transactivator related in sequence to the Mls genes of mice (Albrecht *et al.*, 1992a) and ORF 15 is related to the complement regulatory protein CD59. The area between ORF 69 and ORF 73 also contains no open reading frames in AlHV-1, however in OvHV-2 in contains the predicted open reading frame O8.5. This ORF is predicted to encode a 390 amino acid protein, but its possible function is unknown. Non-coding areas in non-repetitive regions of herpesvirus genomes are unusual, however they are also found in the genome of EHV-2 (Telford *et al.*, 1995). Large regions between ORFs 9 and E5, ORFs 13 and 17 and the region between the IL-10 homologue E7 and the right hand end of the genome contain relatively few genes. The genome of OvHV-2 contains two putative tegument proteins at ORF 75 and ORF 3, which are homologous to formylglycineamide ribotide amidotranferase (FGARAT), an enzyme catalysing the fourth step in the *de-novo* synthesis of purine bases (Barnes *et al.*, 1994). FGARAT homologues are present at these positions in AlHV-1, HVS, BoHV-4 and EHV-2, however EBV and KSHV encode just one copy.

Similarly to other gammaherpesviruses, OvHV-2 contains homologues to cellular genes. ORF O2.5 is not present in AlHV-1 and is predicted to encode an IL-10 homologue. The other gammaherpesviruses encoding IL-10 homologues are EBV (BCRF1) (Vieira *et al.*, 1991) and EHV-2 (E7) (Telford *et al.*, 1995). IL-10 homologues are also found in other DNA viruses such as ORF virus (Fleming *et al.*, 1997) and in other herpesviruses such as human cytomegalovirus (Kotenko *et al.*, 2000) and rhesus cytomegalovirus (Lockridge *et al.*, 2000). IL-10 was first known as cytokine synthesis inhibitory factor (CSIF). Cellular IL-10 has both immunosuppressive and immunostimulatory activities (reviewed by Moore *et al.*, 2001). It inhibits the synthesis of pro-inflammatory cytokines such as TNFα, IL-1ß, IFNγ, GM-CSF and IL-6 (Fiorentino *et al.*, 1991) from monocytes and macrophages and also inhibits expression of MHC class II molecules on monocytes. This therefore inhibits antigen presentation by these cells. IL-10 can act as a stimulatory factor for immature and mature thymocytes, mast cells and B cells (Ding *et al.*, 2000). The IL-10 homologue of EBV is 90% homologous to human IL-10 and is thought to have been acquired from the host. EBV IL-10 acts as a predominantly immunosuppressive cytokine, inhibiting cytokine synthesis by monocytes and reducing antigen specific human T cell proliferation by reducing expression of MHC class II (de Waal Malefyt *et al.*, 1991).

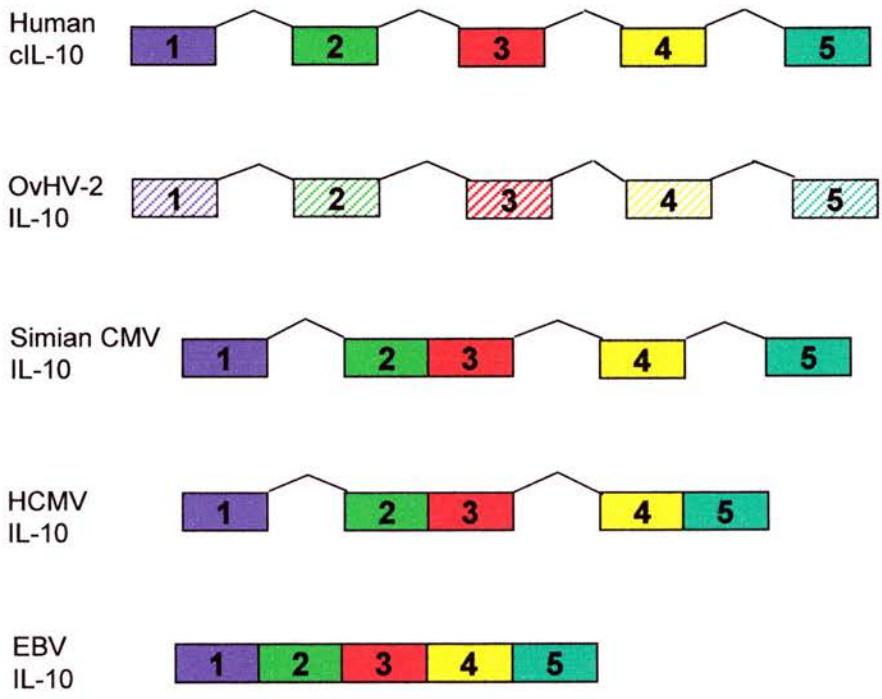
The translated sequence of O2.5 is homologous to both viral and cellular IL-10 protein sequences (Figure 3.2.3.4), however the sequence seems more divergent from cellular IL-10 sequences than either the EBV IL-10 homologue or the ORF IL-10 sequence. The sequence has only 44% homology with the Ovine cellular IL-10 sequence. This is in contrast to the EBV IL-10, which as mentioned is 90% homologous to the human cellular sequence. ORF IL-10 is 80% homologous to ovine IL-10 (Fleming *et al.*, 1997). This IL-10 homologue has retained some of the immunostimulatory activities of cellular IL-10 and can induce proliferation of both thymocytes and mast cells. The HCMV IL-10 homologue is only 27% homologous to cellular IL-10 sequences however it has been shown to have immunosuppressive activity (Spencer *et al.*, 2002). The functional activity of IL-10 proteins is localised to specific regions of the protein (Gesser *et al.*, 1997) and the immunostimulatory activity has been localised to a single amino acid (Ding *et al.*, 2000). The differences

in activity and homology of viral IL-10 molecules as compared to host IL-10 show how the sequences have diverged over time in response to the viral requirements in the host.

Cellular IL-10 genes contain four introns. The OvHV-2 IL-10 is predicted to be spliced and contains 5 predicted exons, in a similar way to cellular sequences. This is in contrast to EBV IL-10, which is not spliced. The predicted splice sites in the OvHV-2 IL-10 homologue are in the same positions as in human IL-10, indicating that these splice sites may be used *in vivo*. It will be of great interest to investigate if these splice sites are used, as this IL-10 homologue would be the only viral IL-10 homologue retaining the intron-exon structure of the cellular gene. The IL-10 homologues of both human and rhesus cytomegalovirus also contain introns (Figure 4.1). The HCMV IL-10 contains two introns, however there is no intron between exons 2 and 3 or between exons 4 and 5. The rhCMV IL-10 contains three introns. The only difference from the splicing arrangement of cellular IL-10 is that there is no intron between exons 2 and 3. It was suggested by Lockridge *et al.*, (2000) that intron-exon arrangement of the CMV IL-10 homologues results from the acquisition of a cellular IL-10 gene by a progenitor CMV followed by sequence variation and loss of introns during speciation. This could suggest that the OvHV-2 IL-10 is a relatively recent acquisition and that introns may be lost in time. The OvHV-2 IL-10 homologue, if functional, could play an important role in aiding persistence of OvHV-2 in the host, possibly by reduction in the host inflammatory response and reduction in presentation of OvHV-2 antigens to the host by down regulation of MHC class II expression. It was observed by Schock *et al.*, (1998) that OvHV-2 infected bovine cell lines express IL-10. RT-PCR analysis of these cell lines using primers specific for OvHV-2 IL-10 would be necessary to determine of the cell lines express viral IL-10.

The genome of OvHV-2 encodes two bcl-2 homologues, located at O4.5 and O9. The ORF O4.5 has also been recently identified in the same position in AlHV-1, although it was not identified in the original complete sequence. The ORF is homologous to E4 of EHV-2, E4 of PLHV-1(Goltz *et al.*, 2002) and BALF1 of EBV

Figure 4.1 The Intron-Exon Arrangement of Viral Spliced IL-10 Homologues



The intron-exon structures of the viral IL-10 homologues of Simian cytomegalovirus (CMV), human cytomegalovirus (HCMV), Epstein-Barr virus (EBV) and OvHV-2 are shown as compared to human cellular IL-10 (cIL-10). The exons are shown as coloured boxes and the exons numbered 1 to 5. The introns are shown as lines joining the exons together. Cellular IL-10 contains 5 exons, whilst in simian CMV IL-10, exons 2 and 3 are joined together. In HCMV IL-10 exons 2 and 3, and exons 4 and 5 are joined together. The intron-exon arrangement of OvHV-2 is only predicted and is shown in shaded in lines.

(Bellows *et al.*, 2002). The O9 gene shares 50% amino acid identity with A9 of AlHV-1 and is homologous to a number of cellular bcl-2 sequences. All gammaherpesviruses sequenced so far encode viral bcl-2 homologues, including HVS (ORF 16), KSHV (ORF 16), MHV-68 (M11) and BoHV-4 (ORF 16). The bcl-2 homologues of HVS (Nava *et al.*, 1997), KSHV (Cheng *et al.*, 1997) and MHV-68 (Roy *et al.*, 2000) have been shown to be functional and it is thought that bcl-2 homologues are therefore important to the viral life cycle. EBV encodes two bcl-2 homologues, encoded by BHRF1 and BALF1.

BHRF1 of EBV is an early lytic cycle gene that inhibits apoptosis induced by a variety of stimuli and it is thought that it may prevent cell death during viral replication (Henderson *et al.*, 1993). BALF1 has been found to lack anti-apoptotic function, but acts to negatively regulate the EBV anti-apoptotic protein BHRF1 (Bellows *et al.*, 2002). Bcl-2 homologues are defined by the presence of one or more bcl-2 homology (BH) domains as well as a C terminal hydrophobic domain. ClustalW analysis showed that O4.5 contains conserved BH domains, confirming this protein as a bcl-2 homologue. The divergence of the non-primate BALF1 proteins from the primate proteins was illustrated by ClustalW analysis and probably reflects differences in the host. It is not known if the bcl-2 homologues of OvHV-2 are functional. It is possible, however that the O4.5 gene of OvHV-2 might negatively regulate the activity of the bcl-2 homologue O9 in the same way that BALF1 of EBV negatively regulates the activity of BHRF1.

O5 encodes a putative G protein-coupled receptor protein and is homologous to A5 of AlHV-1, E6 of PLHV-1 and to E6 of EHV-2. The E6 gene of EHV-2 is the third putative G protein-coupled receptor protein in EHV-2. Although it has seven membrane spanning domains and sequence motifs characteristic of G protein coupled receptor, it does not have high homology to other G protein coupled receptors. No positional homologues of O5 are found in HVS or in KSHV, although G protein-coupled receptor homologues are found in these viruses at ORF 74.

The ORF 74 gene of HVS encodes a functional IL8 receptor (Ahuja & Murphy, 1993) and similarly, the ORF 74 gene of KSHV binds a range of chemokines and has been shown to induce cell proliferation (Arvanitakis *et al.*, 1997). The KSHV ORF 74 gene also exhibits transforming activity in 3T3 cells and

is tumourigenic in nude mice, therefore it is thought to be important in KSHV pathogenesis and oncogenesis. GPCR homologues are therefore thought to be important in induction of host cell proliferation by gammaherpesviruses. The E6 gene of PLHV-1 was expressed in the lymph nodes of pigs with post transplantation lymphoproliferative disease (Goltz *et al.*, 2002), therefore the gene could be of importance in the pathogenesis of this disease. The O5 ORF of OvHV-2 might therefore be of importance in the induction of the proliferation of LCLs in culture, and in the induction of lymphoproliferation *in vivo* as is found in MCF.

The arrangement of the genes O6, O7 and O8 is the same as in AlHV-1. The genes A6 and A7 have also been identified in the genome of PLHV-1. O6 of OvHV-2 is predicted to be spliced and encodes three exons. It has been shown that A6 of PLHV-1 is expressed as a spliced transcript in the same way as is predicted for O6. The arrangement of this region is similar in EBV and the ORFs O7 and O8 are in an analogous position to the EBV gene BLLF1, which encodes the envelope glycoprotein gp350 (Hummel *et al.*, 1984). ORF O7 encodes a putative signal peptide, whilst O8 is predicted to encode a glycoprotein. The O7 and O8 ORFs are predicted to be spliced together to form a complete membrane glycoprotein similar to gp350. In a similar function to gp350, the O7/O8 protein may act as a receptor for OvHV-2 viral entry into host cells.

The O3 open reading frame encodes a semaphorin homologue, as does A3 of AlHV-1 (Ensser & Fleckenstein, 1995). Semaphorins are a large family of secreted and transmembrane signalling proteins that regulate axonal guidance in the developing nervous system (Goshima *et al.*, 2002). Semaphorins are also of possible importance in the immune system, as the transmembrane protein CD100 is a class 4 semaphorin expressed by T and B lymphocytes, NK cell, monocytes, and macrophages. CD100 is thought to be involved in B cell development and in T cell activation. Other viruses such as the pox viruses vaccinia virus (Comeau *et al.*, 1998) and fowlpox (Afonso *et al.*, 2000) also encode semaphorin homologues. The vaccinia virus semaphorin homologue A39R binds a receptor found on lymphocytes and induces cytokine production and intracellular adhesion molecule-1 (ICAM-1) up-regulation in monocytes. O3 is homologous to both viral and cellular semaphorins and could have a role in host immune regulation by OvHV-2.

The sequence of OvHV-2 is similar to that of AlHV-1, reflecting the similarities in the disease caused by the two viruses and the fact that although the natural host of the viruses differs, both OvHV-2 and AlHV-1 can cause malignant catarrhal fever in the same susceptible hosts. OvHV-2 contains a number of genes that may be important in disease pathogenesis, including those that are included in the genome of AlHV-1 (and some in PLHV-1) such as the semaphorin homologue encoded by ORF O3, the two bcl-2 homologues encoded by ORF O4.5 and ORF O9 and the potential G protein-coupled receptor encoded by ORF O5. These indicate that in common with other rhadinoviruses the induction of cell proliferation, the promotion of infected cell survival and evasion of the host immune response may be important in the pathogenesis of OvHV-2 infection.

The inclusion of a viral IL-10 homologue potentially provides OvHV-2 with another way of avoiding the host immune response in early viral replication. It remains to be seen however, if these potentially useful genes are functional in OvHV-2 *in vivo*. It would be interesting to investigate the possible function of the yet uncharacterised genes O3.5 and O8.5, which are unique to OvHV-2 and therefore also of possible importance in viral pathogenesis. The sequencing of this large portion of the OvHV-2 genome provides a starting point for further studies on the pathogenesis of OvHV-2. The availability of the sequence means that antibodies can be raised to viral proteins to study the cellular localisation and possible function and the completion of the sequence would provide a starting point for the production of recombinant viruses to enable the study disease pathogenesis in the *in vivo* rabbit model system.

4.4 Characterisation of the O7 and O8 open reading frames

4.4.1 Analysis of The Expression of O7/O8 in OvHV-2 Infected Rabbit LCLs

The open reading frames O7 and O8 were identified close to the centre of the OvHV-2 genome, in a position analogous to that of gp350/220 of EBV. Gp350/220 functions as a receptor for EBV entry into host cells and it was hypothesised that O7 and O8 might have a similar function in OvHV-2. Work was undertaken to further

characterise the expression of O7 and O8 in OvHV-2 infected cell lines, in order to gain an insight into the possible function of O7 and O8. It was hypothesised that O7 and O8 might be spliced together to form domains of one glycoprotein, therefore initial experiments aimed to determine if this was the case. The cellular localisation of O7 and O8 were also studied using an anti-O8 antibody raised in rabbits, and using an anti-haemagglutinin antibody to study expression of a haemagglutinin epitope tagged O7/O8 construct.

Sequence analysis of the O7/O8 region was performed in order to predict the basic properties of the O7/O8 product as a starting point for experimental analysis. Comparison of the translated sequence with other protein sequences showed that the O7 and O8 proteins had greatest homology with A7 and A8 of AlHV-1, however the sequence also had homology with A7 and A8 of the porcine gammaherpesvirus PLHV-1, showing that these three viruses are highly related. The areas of homology with A7 and A8 were located in distinct blocks, suggesting these areas might be important functionally. The predicted O7 open reading frame was preceded by two possible TATA sequences, but no putative polyadenylation sites were identified. The predicted O8 open reading frame was not preceded by any putative promoter sequences, however a potential polyadenylation signal was identified downstream of the open reading frame. This suggested that the O7 and O8 open reading frames might be spliced together and expressed on the same transcript. The predicted O7 and O8 open reading frames were not in frame with each other. In addition, the end of the O8 sequence was not in frame with the start of O8, therefore splicing would be required for an O7/O8 protein to be expressed from these open reading frames. Analysis of the sequence identified the presence of multiple potential splice sites in the O7 and O8 sequence, some of which could be used to splice the sequences together.

The O7 and O8 translated sequences contained potential N and O glycosylation sites. Hydropathicity analysis revealed a predicted hydrophobic domain in the N terminal region of the O7 protein and on the C terminal region of the O8 protein. The O7 protein also contained a predicted signal cleavage site. The presence of a putative signal sequence on the N terminus of O7 and a putative membrane anchor sequence on the C terminus of O8 suggested that the two ORFs

might be domains of one virion glycoprotein. It was hypothesised therefore from this analysis that the O7 and O8 ORFs formed separate domains of a glycoprotein. The predicted O7/O8 glycoprotein contained an N terminal signal sequence, a C terminal membrane anchoring sequence and glycosylated O8 ectodomain. The ectodomain also contained proline, serine and threonine rich regions.

The predicted features of the O7/O8 gene product are those of a type I virion glycoprotein. A glycoprotein gene is present in this position in all rhadinovirus genomes sequenced so far. All encode a hydrophobic signal sequence, a serine and threonine rich ectodomain, a hydrophobic membrane anchor and a short cytoplasmic tail. The open reading frame of gp350 is spliced and the protein gp220 is expressed in the same frame as gp350. The KSHV equivalent gene, K8.1 is also expressed as two differentially spliced transcripts termed K8.1α and K8.1ß. The transcripts share the same splice donor but employ different splice acceptor sites.

The predicted features of O7/O8 were used as a basis for experimental work to further characterise the open reading frames. Since the O7 and O8 open reading frames were predicted to be expressed on the same transcript and multiple ways of splicing of the transcript were predicted, the expression of O7 and O8 encoding transcripts was analysed. Expression of the RNA encoding the putative O7/O8 glycoprotein in OvHV-2 infected rabbit cell lines was analysed by northern blotting. Northern analysis of total RNA was performed using an OvHV-2 O8 probe, expression of an O8 transcript was not detectable in the cell lines by northern analysis and treatment of the OvHV-2 infected rabbit cell lines with sodium butyrate did not increase expression to the level of detection. Probing the membrane with a probe for the housekeeping gene GAPDH showed that this transcript was easily detectable. Northern analysis of poly A RNA extracted from an OvHV-2 infected rabbit cell line was performed, however it was still not possible to detect expression of O8. It was however possible to detect expression of O8 in RNA extracted from a cell line transfected with a plasmid encoding O7 and O8, which was analysed alongside the BJ/880 RNA as a positive control.

The inclusion of positive controls in the northern analysis showed that the failure to detect expression of O8 by northern analysis was because levels of O8 transcripts in the OvHV-2 infected cell lines were low and not because there was no

RNA on the membrane. It may be that levels of transcripts were low because expression was low, or because the transcripts were unstable and were rapidly degraded once produced. Sodium butyrate is very efficient at increasing viral lytic gene expression in KSHV, however the fact that this did not have any detectable effect in OvHV-2 infected rabbit cell lines suggests that removal of transcriptional repression by chromatin condensation is not sufficient to increase expression of lytic cycle genes to the level of detection.

The low levels of O8 transcripts in OvHV-2 infected rabbit cells suggested that more sensitive methods of detection of the transcripts were required; therefore RT-PCR analysis of the expression of O7 and O8 was performed. The primers used for PCR amplification were designed to amplify the area between the predicted start of the O7 open reading frame and the predicted end of the O8 open reading frame. It was hypothesised that PCR amplification of the area between these two primers would be successful if the O7 and O8 open reading frames were spliced together as predicted. This region was defined as the "full length" O7/O8 RT-PCR product.

It was not possible to detect the full length O7/O8 RT-PCR product by analysis of total RNA extracted from the OvHV-2 infected rabbit cell line BJ/880. RT-PCR analysis of the O7/O8 region using primers designed to amplify smaller defined areas of O7 and O8 confirmed expression of all five regions of O7 and O8 amplified. Two of the primer pairs spanned regions predicted to be spliced, these were the region from O7 to O8 and the region at the end of O8. The RT-PCR product from amplification of the region from O7 to O8 was not easily detectable by agarose gel electrophoresis. Southern analysis of this transcript using the cosmid C57 as a probe confirmed the size of this RT-PCR product and the lack of product in the control sample that omitted reverse transcriptase enzyme. The RT-PCR products were the same length as RT-PCR products produced by analysis of genomic DNA. This meant that there was no evidence of splicing of the O7/O8 RNA. RT-PCR analysis of poly A RNA extracted from an OvHV-2 infected rabbit cell line identified expression of the O7/O8 RNA as a full-length transcript, however the size of this RT-PCR product was the same as the product from amplification of genomic DNA. This suggested that the transcript was not spliced.

The RT-PCR product spanning the area from the O7 ORF to the O8 ORF was difficult to detect compared to PCR products spanning other regions of O7 and O8, this may be because the PCR reaction was inefficient. The fact that a product was detected however, was evidence that O7 and O8 are expressed on the same RNA transcript. The detection of expression of the full length cDNA encoding O7 and O8 by analysis of poly A RNA was further evidence that O7 and O8 are expressed on the same mRNA. In both cases the cDNAs detected were of the same length as product from PCR amplification of positive control genomic DNA, therefore there was a possibility that the products were a result of contamination of the RNA samples with genomic DNA. This is unlikely however, as RNA samples were treated with DNaseI before analysis and control RNA samples without RT enzyme used for PCR amplification did not result in the amplification of any PCR product. Sequencing of the full length RT-PCR product resulting from amplification of poly A RNA would have confirmed that this cDNA did encode O7 and O8, however this was not possible as attempts to clone this cDNA into a sequencing vector were not successful. The amplification of this product was however reproducible and a positive control of genomic DNA resulted in the same product under the same conditions. This suggests that the product is likely to be the correct O7/O8 product and not a spurious PCR product. This work has provided two lines of evidence that the O7 and O8 open reading frames are expressed on the same transcript, however there was no evidence that the transcript was spliced in the OvHV-2 infected rabbit cell lines. Low levels of expression of the O7/O8 transcript made analysis in this cell line difficult and it is possible that spliced mRNAs encoding and O7/O8 protein are produced, but were below detectable levels.

4.4.2 Expression of O7/O8 RNA In Transfected Cell Lines

The analysis of O7/O8 expression in OvHV-2 infected rabbit cell lines was made difficult by the low levels of expression of O7 and O8 in these cell lines. In order to increase expression of O7 and O8, two different expression constructs containing a haemagglutinin (HA) tagged O7/O8 under the control of a CMV promoter were transfected into mammalian cell lines. A HA tagged expression construct of O7 and O8 was made because commercially available antibodies could be used to detect expression of a haemagglutinin epitope tagged protein.

Transfection of the O7/O8 construct pVRO7/8HA into HEK293 cells resulted in the expression of O7/O8 RNA transcripts that were detectable by northern analysis. RNA species of size approximately 2.2kb, 2.4kb and 3kb were observed. RT-PCR analysis of the RNA resulted in a major band of approximate size 2.2kb, a product of size 3kb and a number of smaller products. Cloning of the RT-PCR product into a TA vector for sequencing resulted in the identification of two different spliced O7/O8 cDNAs. The cDNA O7/8-293.11 contained one splice from within the O8 ORF. This splicing pattern meant that the beginning and end of the O8 protein were in frame, however there were stop codons in the cDNA between the predicted O7 and O8 open reading frames.

The cDNA O7/8-293.6 contained a splice within the O8 ORF. This used the same 3' splice acceptor site as seen in O7/8-293.11, however an alternative splice donor site 129 bp 3' to the site used in O7/8-293.11 was used. A splice was also seen within the O7 ORF and a splice in the area between the predicted O7 and O8 open reading frames. Similarly to O7/8-293.11, this cDNA contained stop codons between the O7 and O8 open reading frames, however O8 open reading frame was spliced such that the spliced open reading frame was maintained and did not contain stop codons. None of the cDNAs sequenced seemed to have the potential to encode an O7/O8 protein due to the presence of translational stop signals between the O7 and O8 sequences.

It was hypothesised that there could be a number of reasons for incorrect splicing of the O7/O8 construct. The vector pVR1255 contained a CMV intron A sequence, which has been shown to increase expression of cloned genes (Huang & Gorman, 1990, Xu *et al.*, 2001). It was suspected however that the intron A sequence in the vector pVR1255 might affect splicing, therefore the construct was cloned into the vector pcDNA3.1 *Myc*-His. This vector directed expression under the control of a CMV promoter, however it did not contain intron sequences. This construct was used for all subsequent transfections. It was also hypothesised that splicing of the O7/O8 construct could be regulated by cell-type specific factors. Cell type specific regulation of splicing is well documented in the regulation of the expression of cellular genes (Xu *et al.*, 2002), such as the gene encoding the lymphocyte protein CD45 (Virts & Raschke, 2001) and is found in expression of the HCMV immediate

early gene region (Kerry *et al.*, 1995). In order to address the possibility of tissue specific splicing, the construct pcDNAO7/8HA was transfected into the mouse B cell line A20. This cell line was chosen as OvHV-2 has been shown to infect lymphocytes and has been suggested to infect B lymphocytes in sheep (Baxter *et al.*, 1997). RT-PCR analysis of RNA extracted from transfected A20 cells resulted in major RT-PCR products of the same size of 2.2kb. Since this was the same size as the products identified on analysis of transfected HEK293 cells, it was concluded that additional splicing to that found in HEK293 cells had not taken place and that splicing of the O7/O8 construct was still not correct for expression of an O7/O8 protein. This result suggests that B lymphocyte cell specific factors are not required for splicing of the O7/O8 construct. The question of the possibility of cell type specific splicing may not have been completely addressed by this experiment however, as the target cell of OvHV-2 has not been definitively proved. It has been shown that for AlHV-1 in rabbits T lymphocytes are infected (Mushi & Rurangirwa, 1981, Rurangirwa & Mushi, 1982), and it has also been suggested that the target cell for OvHV-2 is the large granular lymphocyte (Reid *et al.*, 1984). In order to completely address this question transfection of T lymphocytes with the O7/O8 construct would be necessary.

It was also hypothesised that species-specific factors may be involved in the correct splicing of the O7/O8 construct. Species-specific control of splicing is not well documented in control of viral gene expression, but is seen in cellular genes such as p53 (Laverdiere *et al.*, 2000). It is also found in expression of the gene encoding ATP11B, a P-type ATPase (Halleck *et al.*, 2002). OvHV-2 is not known to infect human or murine cells, therefore in order to address the possibility of species specific splicing, the MDBK cell line was transfected.

Transfection of the O7/O8 construct pcDNAO7/8HA into MDBK cells resulted in the expression of O7/O8 RNA which was detected by northern analysis as one RNA species of size approximately 2.4kb. RT-PCR analysis of the RNA resulted in a large product of size approximately 2.2kb and several smaller products. Cloning and sequencing of the 2.2kb band identified two different cDNAs that were both spliced. MDBK4, 6 and 12 were the same sequence as the cDNA O7/8-293.11, containing the same large splice within the O8 ORF. MDBK3 contained the same

splice within O8 as MDBK4, 6 and 12. This cDNA also was spliced at the end of O7. The splice donor site was located close to the end of the predicted O7 open reading frame and the splice acceptor site was in the region between O7 and O8. These two spliced cDNAs also resulted in O7/O8 open reading frames containing stop codons. The first splice donor at position 42 and the second splice acceptor at position 466, are used as splice donor and acceptors in the clone O7/8-293.6. It is possible that splice pattern observed in O7/8-293.6 is an intermediate found before the complete splice from 42 to 466 found in MDBK3.

Similarly to the cDNAs identified in HEK293 cells, none of the cDNAs identified from transfection of MDBK cells represented RNA transcripts that could be translated into a complete O7/O8 protein, due to the presence of translational stop codons between O7 and O8. This suggested that bovine cell specific factors were not required for splicing of the O7/O8 construct. The transcripts expressed in MDBK cells did appear different than those found in transfected HEK293 cells, as no RNA species corresponding to the length of an unspliced transcript was found in MDBK cells, whereas in HEK293 cells, transcripts of varying sizes up to approximately 3kb in size were identified. It may be therefore that splicing of the transcript was more efficient in MDBK cells and a higher proportion of transcripts are spliced.

The fact that transcripts encoding both O7 and O8 could be identified in transfected cell lines is evidence that the O7 and O8 sequences are expressed from the same transcript in this system, however the results shown by the splicing patterns are less conclusive. Transfections into two different cell types using two different expression constructs resulted in the identification of cDNAs with the same splicing pattern in O8. The intronic sequence spliced from O8 in the two different splice variants was closely correlated with the area of O8 that was not homologous to A8. This suggested that the conserved sequence is functionally important, and the same region is spliced from the A8 sequence of AlHV-1. Mutations in the intronic sequence would not affect the function of the protein, as long as the splice donor and acceptor sites and the branch point were conserved. This suggests therefore that this pattern of splicing in O8 could be found in viral gene expression *in vivo*. The O7 sequence contains a distinct area that is homologous to A7 and it may be that in the same way, the region of O7 not homologous to A7 is spliced from the sequence. No

putative splice donor sites in the region at the end of the area of O7 homologous to A7 were identified using the NetGene program, however a number of GT motifs, which could act as splice donor sequences were present in the region.

Transfection of the O7/O8 sequence alone is an artificial situation and it is possible that virus specific factors are required for correct splicing of the O7/O8 construct. A possible viral protein involved in splicing is the ORF 57 protein. ORF 57 is homologous to the HSV protein ICP27. In HVS, ORF 57 has been shown to be involved in redistribution of the SC-35 and snRNPU2 components of the spliceosome (Cooper *et al.*, 1999). It is thought that this may be a way of down regulation of expression of intron containing viral genes. This gene product may also be involved in regulation of splicing, therefore future experiments would involve co-transfection of ORF 57 and O7/O8. Splicing has also been shown to be linked to the promoter driving expression of the spliced gene, as splicing of the fibronectin gene differed when driven by different promoters (Cramer *et al.*, 1997). It is thought that the effect of splicing regulators is modulated by the promoter (Cramer *et al.*, 1999). It is possible therefore that splicing of the O7/O8 construct would have been different if the O7/O8 sequence had been under the control of a different promoter than CMV.

In summary, the fact that transcripts encoding both O7 and O8 are expressed in transfected cells is further evidence that O7 and O8 can be expressed on the same transcript in some situations. The fact that in both splices identified in the O8 open reading frame, the intron spliced out was closely correlated with the area of O8 not homologous to AlHV-1 suggests that the conserved region is functionally important. It also suggests that this pattern of splicing might also be found in A8 of AlHV-1. The splicing pattern that might be used to express a protein translated from the O7 and O8 open reading frames has not been determined, as all splices preceding the O8 open reading frame resulted in the introduction of stop codons. It is hypothesised that the O8 open reading frame might be spliced close to the end of the region of homology with O7. Future approaches to test this hypothesis would involve the cotransfection of ORF 57 with O7/O8 and possibly testing the O7/O8 construct under the control of a different promoter.

4.4.3 Analysis of O7/O8 Protein Expression By Immunofluorescence

Immunofluorescence analysis of the O7/O8 transfected MDBK cells was carried out using an anti-haemagglutinin antibody to identity the cellular localisation of the O7/O8 protein in transfected cells. Although no transcripts were identified that were likely to encode an O7/O8 protein, it was possible that undetected transcripts were translated into O7/O8 protein. Immunofluorescence analysis of the triple HA tagged O7/O8 construct in O7/8 HA transfected MDBK cells did not result in detection of expression of the O7/O8 protein, although fluorescence of the two control expression constructs JκHA and IRF7HA was identified in the paraformaldehyde fixed samples.

Haemagglutinin epitope tagging was chosen as a method of detecting the O7/O8 protein because it was known to be reliable and good commercially available antibodies could be used for detection of the O7/O8 protein. The influenza haemagglutinin epitope tag placed at the 3' end of an expressed protein has been found not to affect protein function or expression (Sells & Chernoff, 1995). A triple haemagglutinin tag was used as this has been found to increase sensitivity of detection (Nakajima & Yaoita, 1997). Efforts were underway to raise an antibody to O8 in rabbits and it was expected that results from the study of transfection of the O7/O8 construct might correlate with results from study of the anti-O8 antibody in OvHV-2 infected cells.

Visualisation of the control HA tagged plasmids suggested that the antibodies were working and the fixation of the cells was being carried out correctly. Since the HA tag was located at the 3' end of the O7/O8 construct, expression of a full length O7/O8 protein was required for detection of the expression using the anti-HA antibody. Failure to detect the O7/O8 protein using the anti-HA antibody suggests that the putative full length O7/O8 protein was not expressed in the O7/O8 transfected cells. This is likely to be due to the fact that the O7/O8 encoding cDNAs detected in O7/O8 transfected cells were not spliced in a way that maintains a complete O7/O8 open reading frame, therefore a full-length protein could not be translated from these transcripts.

It was hypothesised that correctly spliced RNAs may be present in the cells at levels undetectable by RT-PCR and that these RNAs may be translated into detectable amounts of HA tagged O7/O8, however this does not seem to be the case.

Studies of the expression of the O7/O8 protein seem to confirm that the spliced RNAs detected cannot be translated into an O7/O8 protein and splicing of the O7/O8 RNA to result in an in-frame O7 and O8 open reading frame would be required for expression of the protein. Due to the location of the HA tag at the 3' end of the construct, only expression of the full-length protein could be detected. An antibody raised against O7, or the use of a 5' HA tagged construct would be necessary to detect expression of proteins from the O7/O8 open reading frame that did not contain the 3' end of O8.

4.4.4 Generation of an Anti-O8 Antibody in Rabbits

Efforts were made to raise an antibody to O8 in rabbits in order to study the cellular location and expression of the O8 protein in OvHV-2 infected cells. A 207 amino acid region of OvHV-2 O8 was expressed as a GST fusion protein in *E. coli*, in order to immunise rabbits. The O8-GST fusion protein was found to be insoluble and was therefore purified from bacteria as an inclusion body preparation. The inclusion body preparation was then used to immunise rabbits. Sera from the immunised rabbits were tested for the presence of antibodies to the O8-GST fusion protein by western blotting analysis and were found to contain antibodies specific for the O8-GST fusion protein. The immune rabbit serum was used in immunofluorescence analysis of OvHV-2 infected rabbit cells.

A large proportion of the cells analysed displayed positive fluorescence as compared with that observed using pre-immune rabbit sera. The fluorescence pattern observed was intracellular and some cells appeared to display fluorescence round the cell surface. Control non-infected rabbit lymphoblasts were subjected to immunofluorescence analysis using the pre-immune and immune rabbit sera. Moderate levels of fluorescence were seen on a number of cells incubated with both the immune and non-immune sera. Fluorescence was also observed in a small number of cells incubated with PBS followed by the secondary anti-rabbit FITC.

The fact that the O8-GST fusion protein was insoluble meant that it was not possible to purify the fusion protein from the rest of the bacterial proteins by affinity chromatography on immobilised glutathione. This was not too problematic however, as an inclusion body preparation can contain over 90% pure foreign protein and SDS PAGE analysis of the inclusion body preparation showed that although it did contain

other bacterial proteins, it was composed mostly of the O8-GST fusion protein. Protocols are documented involving the re-folding and solubilisation of inclusion body preparations, however inclusion body preparations containing denatured proteins are known to invoke good antibody responses, therefore further purification of the inclusion body preparation was considered to be unnecessary.

The fluorescence observed following incubation of the OvHV-2 infected cells was preliminary data suggestive that a large proportion of cells of the BJ/880 OvHV-2 infected rabbit cell line were expressing the O8 protein. Although fluorescence was observed after incubation with pre-immune serum, this was not of the same intensity as that seen after incubation with the immune serum. The fluorescent staining observed in the PHA treated lymphoblasts, although only seen in a small number of cells, must however be considered.

A number of problems could arise as a result of using a rabbit antibody for indirect immunofluorescence analysis of rabbit cells. The secondary antibody used was an anti-rabbit FITC, therefore this could cause non-specific fluorescence by binding to any rabbit immunoglobulin present on the cells. This would be of particular relevance to the non-infected control lymphocytes, as these cells were likely to include B as well as T lymphocytes and B lymphocytes would carry rabbit immunoglobulin on their surface. The rabbit immunoglobulins in the rabbit sera would bind to Fc receptors, which would be present in and on the surface of rabbit B cells. Both these problems would cause non-specific fluorescence in the rabbit lymphocytes and binding of the anti-rabbit FITC to rabbit immunoglobulin might have been the cause of the fluorescence observed on incubation of the rabbit lymphocytes with PBS and the anti-rabbit FITC. It was originally intended to use the immune rabbit serum for immunofluorescence analysis of OvHV-2 infected bovine cell lines as well as rabbit cell lines, however a bovine cell line was not available at the time of analysis.

The cellular localisation of the O7/O8 protein was predicted to be similar to that of other viral envelope glycoproteins such as EBV gp350 and MHV-68 gp150, therefore a cell surface and Golgi pattern of expression was expected. The fluorescence pattern observed could be consistent with this, however further localisation of the fluorescence using organelle specific staining would be necessary

to determine the cellular localisation of the O8 protein. Evidence of expression of O8 in a high proportion of OvHV-2 infected rabbit cells is consistent with the fact that OvHV-2 infected rabbit cells transmit virus very efficiently, as the O8 protein is predicted to be important in viral infectivity.

In order to determine the molecular weight of the O7/O8 protein, western blotting analysis was performed on lysates of OvHV-2 infected rabbit cell line using the rabbit anti-O8 immune serum. Three bands were identified in samples incubated with immune serum but not in samples identified with pre immune serum. In samples incubated at serum concentrations of 1/100, 1/200 and 1/500, a band was present at approximately 53KDa and two similar sized bands were present at approximately 37 and 38KDa.

The size of the proteins identified is considerably smaller than the size that would be predicted for an O7/O8 protein. The predicted size of the O7/O8 protein was predicted to be approximately 80 - 98KDa depending on how the O8 open reading frame was spliced. The actual molecular weight observed is likely to be higher as the protein is predicted to be glycosylated. The predicted product of the unglycosylated O8 open reading frame alone is 52 – 84KDa depending on how the transcript is spliced. It is unlikely therefore, that the proteins detected represent the O7/O8 protein. It is possible that the 53KDa band might represent an O8 protein. This might be identified following cleavage of the signal sequence from the N terminal end of the O7/O8 protein. It may also be that O8 is expressed as a single protein without O7, however sequence analysis did not identify a promoter sequence preceding O8 or a signal sequence in the protein. The locus on the genome occupied by the O8 ORF would predict that the protein encodes a membrane glycoprotein, which would be expected to contain a signal sequence.

Further analysis would be required to determine the molecular weight of the putative O7/O8 glycoprotein. Further optimisation of this system including the use of non-infected control cells would be required to determine the molecular weight of the O7/O8 protein using the anti-O8 immune serum. It was expected that the use of anti-haemagglutinin antibody on western analysis of cells transfected with a haemagglutinin tagged O7/O8 construct would confirm results obtained using the anti-O8 antiserum, however this method of analysis was also not successful.

4.4.5 Study of The Antibody Response to O8 in Sheep and Cattle

Sheep are thought to be the natural host of OvHV-2 and it is known that they transmit disease to susceptible hosts. It is hypothesised therefore that OvHV-2 undergoes lytic replication in sheep. It has been shown by immunoblotting analysis that sheep sera react to AlHV-1 polypeptides (Herring *et al.*, 1989); this suggests that sheep develop an antibody response to some OvHV-2 proteins. The glycoprotein gp350 is the major antigenic determinant of EBV and absorption of immune sera from EBV infected patients with gp350 greatly reduces their virus neutralising capacity (Thorley-Lawson & Poodry, 1982). In addition the KSHV glycoprotein K8.1 is recognised by human sera on western blotting analysis of KSHV infected cells. Both these glycoproteins are in an analogous position in the genome to OvHV-2 O8, therefore it was reasonable to expect that infected sheep might raise an antibody response to O8.

In order to determine if sheep develop antibodies to the O8 protein, sera from adult sheep, lambs, MCF recovered cattle and uninfected cattle were tested for the presence of antibodies to the O8-GST fusion protein by western blotting analysis. All sera appeared to react weakly with a 52 KDa band at the position of the GST fusion protein, however the fact that uninfected cattle reacted indicated that the band was caused by non-specific binding of the sera to the O8-GST fusion protein. This meant that there was no evidence that MCF recovered cattle, or adult sheep, have specific antibodies to the region of O8 tested.

Sheep sera reacted to AlHV-1 antigens when used at a dilution of 1/40 (Herring *et al.*, 1989). Serum concentrations in these experiments were initially 1/50. Sera were also tested at a concentration of 1/20 and 1/10, however no specific antibody binding was seen. The presence of non-specific antibody binding by the sera used was a problem in these experiments and the non-specific binding may have masked a weak antibody response. Various approaches to reduce the non-specific antibody binding were attempted. These included titration of the secondary and tertiary antibodies, use of Tween 80 at a concentration of 0.5% in both the blocking and the hybridisation buffer and use of a "high salt" hybridisation and blocking buffer, however these methods did not reduce the non-specific binding. The non-

specific antibody binding was however, quite weak and it was considered that this did not detract from the fact that none of the sera contained specific antibodies to the section of O8 tested.

The lack of antibody response to the O8 protein could be for a number of reasons. It is not known if OvHV-2 viral replication occurs in MCF affected cattle, as they do not transmit disease to other animals, therefore cattle may not develop antibodies to O8 for this reason. If viral replication does occur in OvHV-2 infected sheep it may be that this occurs only at low levels insufficient to stimulate a detectable antibody response. This however seems unlikely since, as mentioned, Herring *et al.*, (1989) demonstrated the presence of antibodies in sheep sera which cross-reacted with a number of AlHV-1 antigens and it was assumed that these antibodies were directed against OvHV-2 antigens.

It may be that the region of O8 chosen for expression is not an antigenic determinant of O8 during infection. The region of O8 chosen for expression as a GST fusion protein was predicted to contain antigenic sites (Figure 3.3.1.10), and contained predicted N and O glycosylation sites (Figure 3.3.1.4 & 5). These features indicated that the region might be located outside the viral envelope and possibly expressed on the surface of OvHV-2 infected cells, however the prediction may have been incorrect. It is possible that a different region of O8 may have been recognised by the sera. It is also possible that the O8 protein itself is not found in the viral envelope in a similar manner to EBV gp110, which is found in large amounts in the nuclear membrane, but only in small amounts in the viral envelope (Gong & Kieff, 1990). Further experiments to clarify the situation would therefore involve expression of a different region of the O8 protein, or possibly expression of the whole of the putative ectodomain region in order to raise antibodies to different regions of the O8 protein.

4.5 Conclusions

The study of OvHV-2 pathogenesis remains of importance, as the virus is the cause of MCF in the UK and worldwide. MCF is a devastating disease that is reported sporadically, however outbreaks of economic importance are reported with reasonable frequency. The identification of Caprine herpesvirus-2 as a cause of MCF

provides another member of the family of MCF causing viruses and comparative study of the mechanisms of pathogenesis of these viruses will be of great interest.

This work describes significant advances in the study of OvHV-2 pathogenesis. Cloning and sequencing of much of the virus genome has confirmed OvHV-2 as a gammaherpesvirus similar to AlHV-1. It has also been established that despite being similar to AlHV-1, OvHV-2 contains significant differences that confirm that this is a gammaherpesvirus distinct from AlHV-1. OvHV-2 and AlHV-1 have been shown to be similar to the porcine gammaherpesvirus PLHV-1. Further sequence data of PLHV-1 would be expected to reveal interesting similarities between these three viruses.

The sequence data will prove to be a valuable resource for further study of OvHV-2 and will enable the generation of reagents for further study of virus pathogenesis, such as antibodies and *in situ* hybridisation probes to study *in vivo* expression of viral genes. Little is known about the *in vivo* sites of virus persistence and replication, either in MCF affected animals or in the natural host, the sheep. The genes at the left hand end of the virus genome are expected to be of significant interest as by comparison with other gammaherpesvirus genomes, they are likely to be important in viral pathogenesis. Establishing if the IL-10 homologue encoded by ORF O2.5 is functional will be of particular interest. Work on the OvHV-2 infected lymphoblastoid cell lines has established important data regarding the mode of viral persistence in these cell lines. The finding of evidence of viral replication in OvHV-2 infected rabbit cell lines was followed by the first visualisation of an OvHV-2 capsid.

Study of the O7 and O8 open reading frames provided evidence that these two open reading frames are expressed on the same transcript as predicted, and immunofluorescence analysis using anti-O8 sera raised in rabbits resulted in preliminary data indicating that the O8 protein is expressed in a large proportion of cells in OvHV-2 infected rabbit lymphoblastoid cell lines. This work has provided a starting point for further study of this region.

The lack of a productive tissue culture system for OvHV-2 still remains a problem in the study of this virus. Although evidence of productive cycle replication was demonstrated in rabbit cell lines, the failure to detect transcripts by northern analysis suggests that levels of OvHV-2 transcripts in these cell lines are low. All

OvHV-2 infected lymphoblastoid cell lines were quite short lived, and the availability of cells for analysis was a constraint in some areas of the work. Defining conditions that allow *in vitro* productive replication of this virus would be very useful for the study of OvHV-2 gene expression. Of importance now in OvHV-2 research will be the completion of the viral genome sequence and the establishment of better *in vitro* systems for virus culture. The completion of the virus sequence will be a starting point to gain an insight into the pathogenesis of a gammaherpesvirus that has long remained a mystery.

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Appendix – Published Work

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Ovine herpesvirus 2 lytic cycle replication and capsid production

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Ovine herpesvirus 2 (OvHV-2) causes malignant catarrhal fever in cattle, pigs and deer. We have observed intact circular and linear OvHV-2 genomes in infected T cell lines derived from cows and rabbits. Bovine T cell lines were predominantly latently infected but rabbit T cell lines supported OvHV-2 productive cycle gene expression and virus capsids were demonstrated for the first time.

Malignant catarrhal fever (MCF) is a severe, usually fatal, lymphoproliferative and inflammatory syndrome of domestic cattle, pigs, deer and certain other susceptible ruminants such as bison. Cases of MCF in cattle usually occur sporadically. However, periodically limited epizootic outbreaks occur (Hamilton, 1990) where losses can be substantial. MCF is also the most important virus disease of farmed deer. The disease is mainly caused by either of two bovid gammaherpesviruses that persist subclinically in their natural host. These viruses are highly related to each other and to other gammaherpesviruses such as Kaposi's sarcoma-associated herpesvirus (KSHV), Epstein–Barr virus (EBV) and murine gammaherpesvirus (MHV-68). Alcelaphine herpesvirus 1 (AlHV-1) naturally infects wildebeest and is the source of wildebeest-associated MCF in Africa (Plowright *et al.*, 1960). Domestic sheep are the reservoir of infection for the other main form of the disease, sheep-associated MCF (SA-MCF). The SA-MCF agent, ovine herpesvirus 2 (OvHV-2), is antigenically closely related to AlHV-1 (Herring *et al.*, 1989; Rossiter, 1981, 1983). Limited sequence analysis of the genome corresponding to part of ORF75 shows that OvHV-2 also shares homology at the DNA level with AlHV-1 and other gammaherpesviruses (Baxter *et al.*, 1993; Bridgen & Reid, 1991).

The detection of DNA in diseased animals and inapparently infected sheep has confirmed that OvHV-2 is the aetiological agent involved in SA-MCF (Baxter *et al.*, 1993; Li *et al.*, 1998; Muller-Doblies *et al.*, 1998; Wiyono *et al.*, 1994). OvHV-2 DNA can be found in epithelial tissues and B cells from sheep (Baxter *et al.*, 1997). However, in SA-MCF-affected ruminants, virus DNA has only been observed in hyperplastic T cells (Baxter *et al.*, 1993; Bridgen & Reid, 1991). These T cells can grow in culture into lines and will transmit disease back to cattle as well as to experimental animals such as rabbits and hamsters (Burrells & Reid, 1991; Buxton *et al.*, 1984, 1988). In turn, T cell lines can subsequently be isolated and passaged from these secondary hosts (Reid *et al.*, 1983).

AlHV-1 has been isolated, completely sequenced and will productively infect epithelial cell lines in culture (Ensser *et al.*, 1997; Plowright *et al.*, 1960). In contrast, research on OvHV-2 has been hampered by the lack of reagents and a consistent failure to isolate the virus and define a productive cell culture system. The aim of this work was to ascertain the nature of OvHV-2 genomes in T cell lines and to define a productive culture system for OvHV-2. Here, we show that infected rabbit T cell lines support aspects of the virus productive cycle.

To identify cells supporting productively replicating OvHV-2, we analysed infected T cell lines by Gardella (*in situ* lysis) gel electrophoresis and Southern blotting exactly as described previously (Gardella *et al.*, 1984; Usherwood *et al.*, 1996). This technique discriminates linear from covalently closed circular (CCC) viral genomes which are characteristic of cells supporting either productive or latent herpesvirus infection respectively. OvHV-2-infected T cell lines were derived from an affected cow (BJ/1035) or affected rabbits (BJ/2222, BJ/2223) as described previously (Buxton *et al.*, 1985). The control cell lines, S11 (Usherwood *et al.*, 1996) and BCP-1 (Boshoff *et al.*, 1998), are latently infected with the related MHV-68 and KSHV respectively. Equal numbers (2×10^6) of cells were loaded onto each lane of the gel. A Southern blot of the gel was probed simultaneously with ^{32}P-labelled DNA fragments derived from the OvHV-2 ORF75, the MHV-68 gp150 gene (Stewart *et al.*, 1996) and the KSHV K13 gene. The results are shown in Fig. 1. S11 (MHV-68-infected) and BCP-1 (KSHV-infected) cells contained both CCC and linear genomes but S11 cells contained a higher proportion of linear relative to circular genomes. Indeed, the amount of linear genomes in BCP-1 cells was on the borderline

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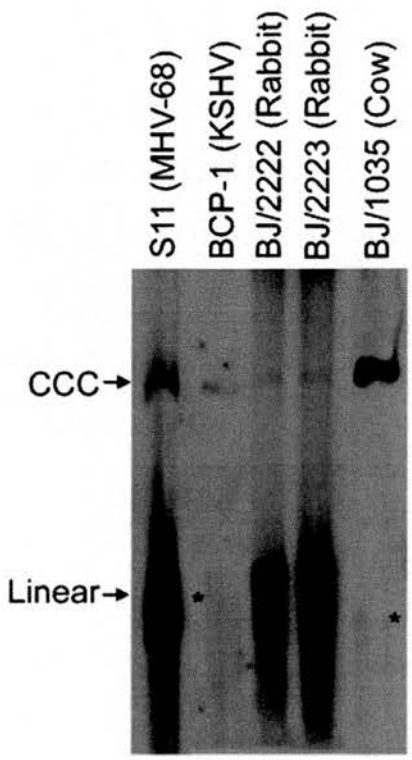

Fig. 1. Gardella gel analysis of lymphoid cell lines. Cells were lysed *in situ* in the agarose gel and then electrophoresed and analysed by Southern blotting. Cell lines used were as indicated above the tracks. The blot was probed simultaneously with ^{32}P-labelled probes specific for MHV-68, KSHV and OvHV-2 followed by autoradiography. The positions of covalently closed circular (CCC) and linear viral DNA are indicated at the left. Asterisks indicate the positions of linear DNA in two cases that were visible on the autoradiograph but did not reproduce photographically.

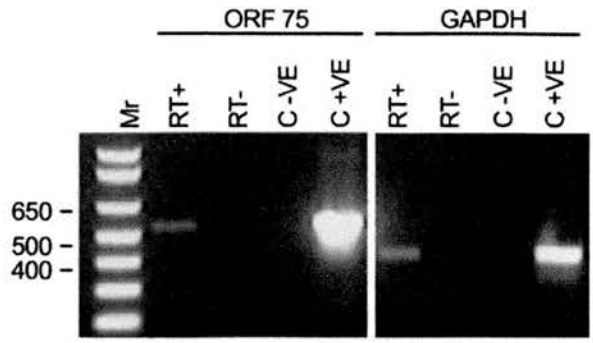

Fig. 2. RT–PCR analysis of OvHV-2-infected rabbit T cell line. RNA was extracted from cells and analysed by RT–PCR using primers specific for GAPDH and ORF75 as indicated above the gel. Products were electrophoresed through 2% agarose gels containing ethidium bromide and visualized using a UV transilluminator. Reactions were performed in the presence (RT+) or absence (RT−) of reverse transcriptase as well as in the absence of template (C-VE). Positive controls (C+VE) of infected cell DNA and mouse cell cDNA were included for the ORF75 and GAPDH primers respectively. Molecular mass determinations were made using a DNA ladder (1 kb plus, Life Technologies) as indicated (Mr). The positions of relevant markers are shown at the left.

of detection (shown by asterisk). This corresponds to a relatively high level of spontaneous reactivation (up to 20% of cells) in S11 (Usherwood *et al.*, 1996), compared with BCP-1 where the level of spontaneous reactivation is only 1–2% of cells (Talbot *et al.*, 1999). An OvHV-2-infected bovine T cell line (BJ/1035) contained predominantly circular genomes and a barely detectable level of linear genomes (shown by asterisk). In contrast, two OvHV-2-infected rabbit T cell lines (BJ/2222 and BJ/2223) contained comparatively large amounts of linear genomes with only a relatively small amount of circular genome apparent. These results strongly suggested that the OvHV-2-infected bovine line that we studied contained predominantly latently infected cells, whereas the two rabbit lines contained latently infected cells but also a relatively large proportion of cells that might be undergoing productive replication.

To determine whether infected rabbit T cells supported OvHV-2 gene expression normally associated with the productive cycle, we performed RT–PCR analysis. Total RNA was extracted from a rabbit T cell line (BJ/2319) or uninfected rabbit lymphoblasts using an RNeasy kit (Qiagen) and then 1 μg was reverse-transcribed using a combination of random hexamer primers (Roche) and Superscript II RT (Life Technologies) as described previously (Roy *et al.*, 2000). RT products were amplified by PCR as described previously (Usherwood *et al.*, 2000), using 40 cycles and primers specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sense 5′ CCTTCATTGACCTCAACTACAT, antisense 5′ CCAAAGTTGTCATGGATGACC) and OvHV-2 ORF75 (sense 5′ GCTTCAGCTTACTCCCTTTAC, antisense 5′ TCAATCAGGTTCAGGTTTACAG). ORF75 in the related EBV and MHV-68 is a late productive cycle gene (Hudson *et al.*, 1985; B. Ebrahimi, personal communication) and is therefore an indicator of productive cycle gene expression. As shown in Fig. 2, a product of the correct size (400 bp) was amplified using GAPDH primers from positive control mouse cDNA and the OvHV-2-infected cell cDNA. No products were seen in control reactions where either RT (RT−) or template (C−) was omitted. Likewise, products 540 bp in length were amplified using ORF75 primers from positive control OvHV-2-infected cell DNA and OvHV-2-infected cell cDNA, but not from negative control samples or uninfected rabbit cells (not shown). Thus, ORF75 was expressed in the OvHV-2-infected rabbit T cell line. This shows that gene expression normally associated with the productive cycle was occurring in the rabbit T cell line. This concurs with the detection of linear genomes in infected rabbit T cell lines.

We surmised that if rabbit T cells contained linear virus DNA and expressed a gene associated with the productive cycle in other gammaherpesviruses then they might produce virus particles. In an initial screen, infected bovine and rabbit T cell lines were examined by transmission electron microscopy. However, after inspection of several hundred cells from

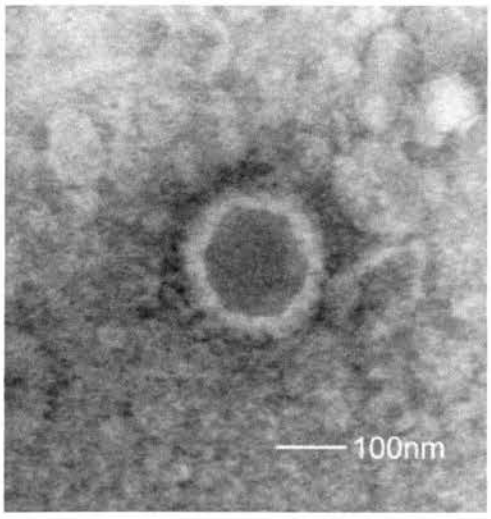

Fig. 3. Transmission electron micrograph of an OvHV-2 particle. Infected rabbit T cells were lysed, the virus pelleted and then visualized by negative staining using transmission electron microscopy.

different preparations of each type of cell line we were unable to detect viral particles. We therefore adopted an alternative approach based upon the concentration of viral particles by pelleting that had proved successful for certain KSHV-infected cell lines. Infected cells were lysed by freeze–thawing and viral pellets were prepared and examined by transmission electron microscopy exactly as described by Arvanitakis *et al.* (1996). Fig. 3 shows that characteristic herpesvirus capsids were observed in extracts from infected rabbit T cells. These appeared to be reminiscent of A-type capsids that do not contain viral DNA and are non-infectious. Thus, infected rabbit T cells not only display markers of the productive cycle but also produce visible capsids. However, the lack of mature capsids and particles argues that the majority of cells are undergoing an abortive productive cycle.

Previous results had implied that OvHV-2-infected T cell lines were capable of producing infectious viral particles because they were capable of causing MCF after experimental transfer into rabbits and hamsters (Burrells & Reid, 1991; Buxton *et al.*, 1984, 1988). While the results presented here represent a significant advance in OvHV-2 research in delineating cell lines that support gene expression that is normally associated with the productive cycle, they still fall short of defining an amenable, fully productive culture system. The presence of linear genomes in cells infected by other gammaherpesviruses is directly correlated with the presence of cells undergoing full productive replication. There are clearly significant numbers of linear viral genomes and evidence of productive cycle gene expression in infected rabbit T cells. However, neither gives an indication of the proportion of cells involved and RT–PCR gives no idea of the levels of gene expression. The inability to detect viral particles in intact cells argues for one of two conclusions. Firstly, that only a very small percentage of cells is undergoing productive replication resulting in particles or, alternatively, that there are a larger percentage of cells undergoing a productive cycle that is largely abortive with respect to particle production. These limitations aside, the delineation of cell types maintaining either latency or aspects of the productive cycle will enable the study of these phases of the virus life-cycle in more detail, in particular the delineation of antigens associated with each phase. However, the key to the analysis of this virus is the determination of the complete DNA sequence of the genome. The definition of an *in vitro* culture system supporting productive infection provides the substrate for such a sequencing project.

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